3-1-2013

Identification of cold-responsive genes in a New Zealand alpine stick insect using RNA-Seq.

Luke T Dunning

Alice B Dennis

Duckchul Park

Brent J Sinclair

Richard D Newcomb

See next page for additional authors

Authors
Luke T Dunning, Alice B Dennis, Duckchul Park, Brent J Sinclair, Richard D Newcomb, and Thomas R Buckley

CBP ms.21439 Revised – part D

Identification of cold-responsive genes in a New Zealand alpine stick insect using RNA-Seq

Luke T. Dunning[1,2,3], Alice B. Dennis[1,3], Duckchul C. Park[1], Brent J. Sinclair[4], Richard D. Newcomb[2,3,5], Thomas R. Buckley[1,2,3]

[1]*Landcare Research, Private Bag 92170, Auckland, New Zealand*

[2]*School of Biological Sciences, University of Auckland, Auckland, New Zealand*

[3]*Allan Wilson Centre for Molecular Ecology and Evolution, New Zealand*

[4]*Department of Biology, The University of Western Ontario, London, ON, Canada N6G 1L3*

[5]*The New Zealand Institute for Plant & Food Research Limited, Auckland, New Zealand*

Corresponding Author:

Luke T. Dunning

Landcare Research, Private Bag 92170, Auckland, New Zealand

tel: +64 9 574 4155

Dunningl@landcareresearch.co.nz

Abstract

The endemic New Zealand alpine stick insect *Micrarchus* nov. sp. 2 regularly experiences sub-zero temperatures in the wild. 454-based RNA-Seq was used to generate a *de novo* transcriptome and differentiate between treatments to investigate the genetic basis of cold tolerance. Non cold-treated individuals were compared to those exposed to 0 ^{o}C for 1 h followed by a 1 h recovery period at 20 ^{o}C. We aligned 607,410 Roche 454 reads, generating a transcriptome of 5,235 contigs. Differential expression analysis ranked candidate cold responsive genes for qPCR validation by *P*-value. The top nine up-regulated candidates, together with eight *a priori* targets identified from previous studies, had their relative expression quantified using qPCR. Three candidate cold responsive genes from the RNA-Seq data were verified as significantly up-regulated, annotated as: *prolyl 4-hydroxylase subunit alpha-1* (*P4HA1*), *staphylococcal nuclease domain-containing protein 1* (*snd1*) and *cuticular protein analogous to peritrophins 3-D2* (*Cpap3-d2*). All three are novel candidate genes, illustrating the varied response to low temperature across insects.

Keywords: stick insect,

1. Introduction

Temperature is a critical variable affecting the performance and distribution of insects (Chown and Nicolson, 2004). The New Zealand alpine zone was formed approximately 5 Mya ago during the Kaikoura Orogeny (Batt and Braun, 1999; Batt *et al*., 2000; Chamberlain and Poage 2000). This recent origin has led to substantial adaptive radiation with all major New Zealand insect lineages represented in alpine habitats (Buckley and Simon, 2007); in particular, the incidence of alpine stick insects (order Phasmatodea) is globally unusual (Salmon, 1991). Out of the 23 endemic New Zealand species from 10 genera (Buckley and Bradler, 2010), only *Micrarchus* nov. sp. 2 (Voucher specimen NZAC03009458 from New Zealand Arthropod Collection, Landcare Research, Auckland, New Zealand) is exclusively found at high elevations (600 to 1,409 m above sea level). This species inhabits the mountain ranges of the north-west South Island, which regularly experience sub-zero temperatures throughout the year (Salmon, 1991). All life stages of this species overwinter (A.B. Dennis and L.T. Dunning, unpublished observations), with those alive at the end of the summer capable of surviving through until spring.

At low temperatures all insects need to mitigate problems associated with reduced cell membrane fluidity (Overgaard *et al.,* 2005), changes in ion concentration (Koštál et *al.,* 2007), induction of apoptotic pathways and other non-freezing cold injuries (Bale, 2002). In addition, lower sub-freezing temperatures lead to a risk of internal ice formation, against which cold-hardy alpine insects adopt freeze avoidant or freeze tolerant strategies (Wharton, 2011). In New Zealand, the majority of alpine insects survive by being moderately freeze tolerant with no incidence of freeze avoidance currently recorded (Wharton, 2011). Nothing is known about cold tolerance in any stick insects.

Very little is known concerning the genes underpinning cold tolerance in cold-hardy insects, as most studies focus on chill-susceptible *Drosophila* species (Hoffmann, 2010). However, *Drosophila* studies have identified a number of candidate genes associated with the molecular response to cold. The most prominent include heat shock proteins (Qin *et al*., 2005) and the *Frost* gene (Goto, 2001). A plethora of other genes and proteins have also been identified as up-regulated in response to low temperature in a variety of insects, with functions related to stress, metabolism, cuticles, membranes, gene regulation, cytoskeletal and immune function (Denlinger and Lee, 2010; Teets *et al.,* 2012). Many of those identified do not overlap between species (Qin *et al.,* 2005; Colinet *et al*., 2007; Robich *et al.,* 2007; Clark and Worland, 2008; Li and Denlinger, 2008; Denlinger and Lee, 2010); we expect that this diversity reflects interspecific variation in response to cold, and is not solely a product of differing methodologies. Additionally, cold tolerance is plastic over both short-term (rapid cold hardening; Lee *et al.,* 1987) and long-term (seasonal acclimation) temporal scales. Gene expression is not essential for this plasticity (Sinclair *et al.,* 2007) but underpins long term responses to, preparation for, and recovery

from cold-shock. To fully comprehend the molecular mechanisms underpinning the variation in insect responses to the cold there is a need to extend genome-based studies beyond the Diptera, in particular to include insects that experience sub-zero temperatures in the wild.

High-throughput sequencing (HTS) and RNA-Seq have allowed the investigation of species that are not established genetic models, yet display adaptations that make them pertinent to an array of ecological and physiological questions (Wang et al., 2009). However, RNA-Seq is still to be applied explicitly to cold tolerant insects and their response to low temperature (Storey and Storey, 2012). Methods previously adopted to identify genes differentially expressed as a consequence of low temperature include cDNA library screening (Bilgen *et al.,* 2001), suppressive subtractive hybridisation (SSH; Rinehart *et al.,* 2007; Robich *et al.,* 2007) microarrays (Qin *et al.,* 2005; Laayouni *et al.,* 2007; Sørensen *et al.,* 2007; Purać *et al.,* 2008; Zhang *et al.,* 2011; Teets *et al.,* 2012) and quantitative real-time PCR (qPCR; Clark and Worland, 2008). Although microarrays have the potential to provide whole-transcriptome snapshots, the ability to identify novel transcriptional profiles associated with cold tolerance is limited by the original starting material used to construct a cDNA array (Wang *et al.,* 2009). Even with genome-based arrays (such as those used for *Drosophila*), there is a limited ability to distinguish between splice variants, patterns of allelic expression and to quantify low abundant transcripts (Wang *et al.,* 2009). By applying HTS to insects across a range of cold-tolerance phenotypes it will not only be possible to uncover novel genes but also allow the development of a more complete picture of the complexity and common features that underlie insect cold tolerance.

In this study, we use HTS and *de novo* transcriptome alignment to identify novel candidate genes associated with recovery from cold-shock in *Micrarchus* nov. sp. 2, a cold-hardy stick insect currently lacking genomic resources.

2. Materials and Methods

2.1 Field collections

Alpine *Micrarchus* nov. sp. 2 specimens were collected from Sewell Peak, the Paparoa Range, New Zealand (42^{o} 24.312 S, 171^{o} 20.585 E, elevation 822 m). Samples for HTS at the University of Otago, HTS at Landcare Research and quantitative RT-PCR (qPCR) were collected on the 20/03/2009, 11/02/2011 and 27/01/2012, respectively. At Sewell Peak, temperatures in late summer can still drop to a few degrees above freezing (A.B. Dennis and T.R. Buckley, unpubl. observations). Collections were transported live to Landcare Research, Auckland. They were kept en masse in a vivarium for a minimum period of 3 weeks for acclimatisation in 12:12 light:dark cycle under ambient room temperature and humidity prior to experimentation. A constant diet of freshly collected pōhutukawa (*Metrosideros excelsa*) was provided. All subsequent procedures were performed exclusively on adult females.

106

107 *2.2 Cold-shock treatments*

108 The control groups were snap frozen in liquid nitrogen directly from approximately 20 ^{o}C. Cold-shock
109 experiments were performed immediately afterwards. The stick insects were held at 0 ^{o}C for 1 h in 50
110 mL Falcon tubes that had been pre-immersed in ice baths. A 1 h recovery at approximately 20 ^{o}C
111 followed this treatment. Previous studies associating cold exposure to gene expression have used a
112 similar temperature and recovery period (Goto, 2001; Sinclair *et al*., 2007; Teets *et al.,* 2012). We
113 expect that *Micrarchus* nov. sp. 2 would potentially encounter 0 ^{o}C in the field at any time of year (A.B.
114 Dennis & T.R. Buckley, unpublished observations). All insects survived the treatment and were moving
115 in a coordinated fashion at the end of the recovery period. Insects were then directly snap frozen and
116 stored at -80 ^{o}C.

117

118 *2.3 RNA extraction and cDNA synthesis*

119 All experiments used the head, antennae and prothorax of the animals. These were removed with
120 sterilised scalpel blades and ground in liquid nitrogen using a mortar and pestle. mRNA was extracted
121 using Dynabeads mRNA DIRECT^{TM} Kit (Invitrogen, Carlsbad, CA, USA; cat. no. 610.06) following the
122 manufacturer's protocol, with one amendment; samples were passed through QIAshredder columns
123 (Qiagen, Hilden, Germany, cat. no. 79656) for sample homogenisation and DNA shearing. RNA quantity
124 was determined by spectrophotometry prior to cDNA library construction (Roche, Mannheim,
125 Germany, "cDNA Rapid Library Preparation Method Manual-GS FLX Titanium Series", October 2009
126 (Rev. Jan 2010)). Individual libraries were tagged with MID-labelled primers during preparation. cDNA
127 Libraries were sent to the University of Otago High-Throughput DNA Sequencing Unit for purification
128 and sequencing on a Roche 454 GS FLX sequencer.
129 To increase the number of reads for *de novo* transcriptome assembly, two additional samples were
130 sequenced at Landcare Research (Auckland). Total RNA was extracted using the TRIzol (Invitrogen, cat.
131 no. 15596-018) RNA isolation method, followed by mRNA purification using the Oligotex mRNA Spin-
132 Column Protocol (Qiagen, Hilden, Germany, cat. no. 70022). Quality and concentration of RNA
133 extractions was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies,
134 Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). cDNA
135 libraries were constructed using the cDNA Rapid Library Preparation Manual Method (Roche,
136 Mannheim, Germany, GS Junior Titanium Series, May 2010 (Rev. June 2010)). Individual libraries were
137 tagged with MID-labelled primers during preparation. Library quality was assessed using the Agilent
138 2100 Bioanalyzer and TBS 380 Fluorometer (Turner Biosystems, Sunny-vale, CA, USA). Sequencing was
139 performed on a 454 GS Junior (Roche, Mannheim, Germany).

For qPCR samples, total RNA was extracted using TRIzol (Invitrogen, cat. no. 610.06) according to the manufacturer's protocol. A further RNA clean-up was performed using the RNeasy Mini Kit (Qiagen, cat. no. 74104). Quality and concentration of RNA extractions were assessed using a NanoDrop ND-1000 spectrophotometer. Contaminating DNA was removed using the Ambion DNA-*free*™ DNase Treatment kit (Invitrogen, cat. no. AM1906). The first strand cDNA synthesis used the SuperScript III First Strand Kit (Invitrogen, cat. no. 18080-051). Successful DNase treatment and cDNA preparation were verified using post and pre cDNA synthesis samples as a template for PCR. Glyceraldehyde 3-phosphate (G3P, F1 = 5'-TGCCAGGCAGTTGGTGGTGC-3', R1 = 5'-ATTCGGCCGCATCGGTCGCC-3'), which amplifies products from both genomic DNA and cDNA (442 bp), was used to check for successful removal of genomic DNA from pre cDNA synthesis templates. Enolase primers (ENO, F1 = 5'-AGCACTACCACGGAAAGGGGGT-3', R1 = 5'-ACCATGGTGCCCCAGCCATT-3') amplify an approximately 1,000 bp product from cDNA only and successful amplification was an indication of cDNA quality. PCRs consisted of 2.5 µL of 2mM dNTP (Roche, Mannheim, Germany), 1 µL of 10 µM bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA), 2.5 µL of 10X FastStart *Taq* DNA Polymerase PCR Buffer with MgCl2 (Roche), 1.5 U FastStart *Taq* DNA Polymerase (Roche) and 10 pmol of the forward and reverse primers (Sigma-Aldrich) in a total volume of 25 µL. Amplifications were performed on a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) using the following parameters: 5 min at 95 ^{o}C; 40 cycles of 1 min at 94 ^{o}C, 1 min at 60 ^{o}C and 1 min at 72 ^{o}C; and 10 min at 72 ^{o}C. Successful amplification was assessed by gel electrophoresis on 1% agarose gels containing 0.5 mg mL^{-1} ethidium bromide.

2.4 454 transcriptome alignment

Sequences were cleaned, trimmed and aligned using Newbler GS De novo Assembler (V. 2.5.3). Redundancy within the alignment was removed using the Geneious (Drummond *et al*., 2012) *de novo* assembler with two different sequence similarity cut-offs (custom sensitivity with default parameters, minimum overlap = 40 bp and sequence similarity = 100% or 90%). Reassembled Isotigs (putative genes and their splice variants) are subsequently referred to as contigs and were manually checked to assess validity and to ensure they were not the result of repetitive DNA (e.g. microsatellites).

The alignment was annotated using Blast2GO (Conesa *et al.,* 2005) on the 4th of September 2011 against the nr and SwissProt databases (minimum e-value cut-off < 1^{-10}). To identify non annotated contigs selected for qPCR verification from the differential expression (DE) analysis, the contig sequences were searched against an unpublished Illumina transcriptome from the same species (L.T. Dunning *et al.* unpublished). Any matching Illumina contigs that were longer were subsequently searched against the SwissProt and nr databases.

2.5 Sequence counts and differential expression

Sequence counts for each individual contig were generated by mapping the Newbler-trimmed data back onto the alignment using PanGEA (v. 1.04) (Kofler *et al.,* 2009). Reads retained for further analysis had to map unambiguously to one contig, have a minimum alignment length of 40 bp, incorporate >20% of the read length and a minimum sequence similarity of 85%. To be considered for subsequent DE analysis each contig had to have a combined total of at least ten counts.

DE analysis in R (V. 2.13.0) (R Development Core Team, 2012) used DESeq (V. 1.4.1) (Anders and Huber, 2010), edgeR (V. 2.2.5) (Robinson *et al*., 2010) and baySeq (V. 1.6.0) (Hardcastle and Kelly, 2010) packages. DESeq and edgeR are similar exact test approaches, whereas baySeq employs a Bayesian method to assign likelihoods to specified models. All methods use the negative binomial distribution that compensates for higher levels of biological than technical variation. Raw counts were normalised for library size using the *calcNormFactors* and *estimateSizeFactors* functions in edgeR and DESeq, respectively. The quantile normalised counts from edgeR were used for subsequent analysis in baySeq. In edgeR both common and the more stringent tagwise dispersion methods for estimating DE were used. Two models were specified for analysis in baySeq; equal expression between all samples and differential expression between the control and cold-shocked treatments. baySeq calculated DE with and without transcript length as an additional normalisation factor. Analysis was performed with 10,000 iterations and 100 bootstraps.

2.6 qPCR verification

The results of the DE analysis were ranked by *P*-value or likelihood depending on the R package used. Subsequent qPCR verification of candidate cold tolerance genes was performed. Eight *a priori* genes (**Table 3**) identified as differentially expressed or with a possible role in cold tolerance from previous studies found in the *de novo* transcriptome were also targeted, along with *alkb5* (selected by eye from corrected sequence counts). In addition, three stably expressed genes identified by DE analysis (*P*-value > 0.8; > 1,000 counts) across treatments were selected as references to normalise relative expression levels among genes. These were: glyceraldehyde-3-phosphate dehydrogenase 2 (*g3p2*), paramyosin long form (*mysp1*) and elongation factor 1-alpha 2 (*ef1a2*). Primers were designed with stringent criteria using the Primer3 (Rozen and Helen, 2000) plugin in Geneious (Drummond *et al*., 2012). All primers had a Tm of 59-61 ^{o}C (except NADH_F1 Tm = 57.62 ^{o}C), GC content between 40-60%, were 18-25 base pairs long and amplify a product between 60-150 bp in length (Supplementary material A). qPCR was performed using LightCycler 480 SYBR Green I Master mix (Roche, Mannheim, Germany, cat. no. 04707516001), 0.25 µM each primer and approximately 5 ng of template cDNA with a total reaction volume of 10 µl. Reactions were carried out on a LightCycler 480 (Roche, Mannheim, Germany) with the default cycling parameters and 60 ^{o}C annealing temperature. Two treatments (control versus cold-shock) with three biological repeats, each with three technical repeats and three negative controls

were, carried out per gene. All genes were analysed in two 384-well plates. A preliminary run and subsequent melting curve analysis was used to exclude genes where more than one product was amplified. Quantification cycle (C_q) values for each reaction were calculated using LinRegPCR (Ramakers *et al.,* 2003). Primer efficiencies were calculated on average for each gene. Technical replicates where the efficiency was greater than 5% of the mean were excluded from subsequent analysis. The most suitable reference genes and normalisation factors were calculated using geNorm (Vandesompele *et al.,* 2002) based on the geometric means. For the first plate *ef1a2* and *mysp1* were selected and for the second plate *g3p2* and *mysp1* were used. An approximate Pfaffl (Pfaffl, 2001) method generated relative amounts of each target gene. The raw relative expression values were natural log transformed and a one tailed t-test used to access significant differential expression between treatments.

3. Results

3.1 Transcriptome generation

Four individually-tagged cDNA libraries, each constructed from the head, antennae and prothorax of a single *Micrarchus* nov. sp. 2, were sequenced on the Roche 454 GS FLX platform producing a total of 524,120 sequences (**Table 1**). This dataset was supplemented with a further 83,112 Roche 454 GS Junior sequences from two cDNA libraries constructed from the head, antennae and prothorax of additional individuals. The resulting 607,410 sequences comprised 291,936,440 nucleotides with a median read length of 482 bp (mean = 481; SD = 112). Raw data were submitted to the National Centre for Biotechnology and Information (NCBI) (Sequence Read Archive (SRA) submission number SRA057228). After trimming to remove bases with low quality scores, adapters and MID barcodes there were 514,993 (84.79%) sequences, 150,267,849 (51.47%) nucleotides and a median sequence length of 313 bp (mean = 292; SD = 99).

An initial Newbler alignment produced 5,827 isotigs longer than 50 bp with a median length of 593 bp (mean = 843; SD 928). These belonged to 4,490 isogroups (putative genes). To remove redundancy the alignment was reassembled using the Geneious *de novo* assembler (Drummond et al., 2012) which reduced the number of contigs to 5,235. This new alignment incorporated 78% of the original sequence data with a median contig length of 588 bp (mean = 819; SD 884). The longest contig was 20,518 bp (BLASTx to SwissProt identified this contig as a cDNA encoding Twitchin). 1,058 contigs were longer than 1,000 bp and 3,240 contigs were greater than 500 bp. Sequence counts were generated by mapping the Newbler trimmed reads back onto the alignment. Between 65 - 67% of raw reads (total = 327,870 sequences) for each sample mapped back to 2,602 contigs that had a combined total at least ten counts; these were used for subsequent differential expression analysis.

The complete *de novo* transcriptome was annotated (with an *E*-value < 1^{-10}) using three approaches: (1) BLASTn against the non redundant (nr) protein database identified 2,630 (50 %) contigs, (2) BLASTx against nr database identified 2,060 (39 %) contigs and (3) BLASTx against the SwissProt database identified 1,991 (38 %) contigs. All contigs are subsequently referred to by their top BLAST match. Nineteen of the 20 most commonly hit species for the lowest *E*-value BLASTx match against the nr database (**Figure 1**) were insects, apart from *Daphnia pulex*. In spite of polyA selection during cDNA library preparation, a substantial number of rRNA sequences remained (17-22% of reads from each sample were identified as 18S and 28S rRNA).

3.2 Expression analysis

Relative mRNA abundances were compared between cold-exposed (1 h at 0 °C followed by 1 h at 20 °C) and control (kept at 20 °C throughout) individuals to identify differentially-expressed candidate cold-responsive genes. Using edgeR with common dispersion two down-regulated contigs were detected (adjusted *P*-value of < 0.05) during recovery from cold-shock. The other tests did not reveal any further candidates with the limited biological replication (n = 2) resulting in high false discovery rates. A relaxation of the stringency to uncorrected *P*-value was permitted as qPCR should identify any false positives. Using edgeR with common dispersion 168 contigs were identified as differentially expressed (94 up, 74 down, *P*-value < 0.05) (**Figure 2**). The edgeR with tagwise dispersion analysis identified 106 differentially expressed contigs (56 up, 50 down, *P*-value < 0.05). DESeq identified 10 differentially expressed contigs (7 up, 3 down, P-value < 0.05). The baySeq analyses identified 2 up-regulated contigs (likelihood > 0.80). A full list of all significant differentially regulated genes can be found in Supplementary material C.

Up-regulated contigs during recovery from cold-shock were ranked by *P*-value, with the top four from each of the five analyses shown in **Table 2** (n = 11 due to redundancy between results). No common contig was identified by the five approaches, but *staphylococcal nuclease domain-containing protein 1* (*snd1*) was in the top four differentially expressed contigs for at least one of the analyses in the three R packages implemented (edgeR, DESeq and baySeq).

3.3 qPCR verification

Of the eleven candidates identified from RNA-Seq differential expression analysis as up-regulated (**Table 2**), nine were verified using qPCR with samples from new biological replicates (n=3) exposed to the same treatments. Of the nine genes, three were statistically significantly up-regulated in cold-exposed individuals (**Figure 3**). These were *prolyl 4-hydroxylase subunit alpha-1* (*P4HA1*, *P*-value = 0.0327), *snd1* (*P*-value = 0.0065) and *cuticular protein analogous to peritrophins 3-D2* (*Cpap3-d2*, *P*-

279 value = 0.0469). None of the eight cold-responsive genes (**Table 3**) selected *a priori* from the literature
280 were significantly differentially expressed (**Figure 3**).
281
282 **4. Discussion**
283
284 This study exploits recent developments in HTS and *de novo* transcriptomics to investigate the genetic
285 basis of cold tolerance in an alpine New Zealand stick insect. This is the first study to apply RNA-Seq
286 specifically to cold-hardy insects and their response to mild cold exposure. No prior assumption or
287 genomic information was required to discover three novel cold-responsive genes.
288 We identified three robust candidate genes that are up-regulated in response to a mild cold shock in
289 *Micrarchus* nov. sp. 2. These genes are *snd1*, *P4HA1* and *Cpap3-d2* and are associated with
290 transcription, amino acid metabolism and cuticular organisation, respectively. As such, these new
291 candidates differ in function from those identified in other insect species. The low number of genes
292 identified in our study may be due to the response by gene expression response to cold being slow,
293 although in *Drosophila* the window of acute gene expression response to cold-shock starts to decline
294 after three hours (Sinclair et al., 2007). We note that few genes with acute responses to cold exposure
295 have been identified in *Drosophila* (Qin *et al.,* 2005), and that longer recovery leads to the up-regulation
296 of different gene sets in that species (Zhang *et al.,* 2011). The limited biological replication (n = 2) for
297 the RNA-Seq and differential expression analysis likely led to a high false discovery rate. However, the
298 conservative two-tier approach of RNA-Seq coupled with subsequent qPCR validation corrects for Type
299 1 error and allows confidence in the three cold-responsive genes we identified. However, it is likely
300 that with further power and a wider range of treatment and recovery conditions, it will be possible to
301 identify additional cold-responsive genes in this species.
302 No orthologues selected *a priori* from the literature showed differential expression in response to cold
303 exposure in *Micrarchus* nov. sp. 2. However, because of the small number of transcriptomic studies
304 outside of Diptera, coupled with few studies using cold-hardy species, we are unable to determine
305 whether this difference is because hemimetabolous insects and Diptera have different responses to
306 cold, or due to 0 ^{o}C being a relatively mild cold stress for an alpine insect. Data loggers (A.B. Dennis &
307 T.R. Buckley, unpublished observations) indicate that temperatures in the microhabitat are quite stable
308 near 0 °C beneath snow pack and that (even in the summer) 0 °C is a temperature experienced during a
309 cold night, suggesting that 0 °C is ecologically-relevant for this species. We are also unable to
310 determine whether the observed changes indicate a repair response to damage, or a preparatory
311 response associated with acclimation to cold. As the RNA-Seq approach becomes more widely
312 adopted, it will be possible to compare cold responses in a phylogenetic context, helping to address
313 some of these questions.

314

315 *4.1 Cold-responsive genes*

316 The s*nd1* gene encodes a complex, six-domain, multifunctional protein with many biological functions
317 (Ying and Chen, 2012; Sundstrom *et al*., 2009). Snd1 is cleaved by caspase-3-like enzymes in apoptosis
318 (Sundstrom *et al*., 2009). Rapid cold-hardening in *Drosophila* reduces apoptosis and lowers caspase-3-
319 like protein levels (Yi *et al*., 2007), and we speculate that elevated expression of *snd1* in cold-shocked
320 *Micrarchus* nov. sp. 2 may be associated with this process. In other organisms the snd1 protein is also
321 associated with the accumulation of mRNA into stress granules (Gao *et al.,* 2010; Weissbach and
322 Scadden, 2012) and the cleavage of double-stranded RNA (Caudy *et al.,* 2003). Thus, it is possible that
323 *snd1* expression is part of a general stress response in *Micrarchus* nov. sp. 2, rather than being
324 specifically cold-related. Further experiments monitoring the response of *Micrarchus* nov. sp. 2 to other
325 abiotic stresses could identify if up-regulation of *snd1* is cold-specific.
326 *PAHA1* encodes an oxidoreductase enzyme catalysing the hydroxylation of proline to hydroxyproline
327 during the maturation of collagen, and possibly other proteins (Mann *et al.,* 1996; Shoulders and
328 Raines, 2009; Gorres and Raines, 2010). The PAHA1 enzyme also modifies proline residues of incorrectly
329 folded collagen after stress in human cells (Vonk *et al.,* 2010). Thus, increased expression of the *PAHA1*
330 gene in *Micrarchus* nov. sp. 2 might be associated with stabilisation or the refolding of proteins that
331 were denatured by the cold-shock treatment, or to stabilise structural peptides in anticipation of future
332 cold exposures.
333 The final robust candidate we identified was *Cpap3-d2*, which has been previously identified in genome
334 sequences of *Tribolium castaneum*, *Nasonia vitipennis* and *Acyrthosiphon pisum* (Jasrapuria *et al*.,
335 2010). *Cpap3-d2* is associated with the epidermal cuticle of *T. castaneum,* and different *Cpap*
336 transcripts appear to affect the rigidity of the cuticle in the elytra and hindwings (Jasrapuria *et al*., 2010;
337 Dittmer *et al.*, 2011). Increased expression of cuticular proteins has been recorded in cold-shocked and
338 diapausing *D. melanogaster* (Qin *et al.,* 2005, Baker and Russell, 2009), after cold exposure in *Aphidius*
339 *colemani* (Colinet *et al.* 2007) and in overwintering *Cucujus clavipes puniceus* larvae (Carrasco *et al.,*
340 2011). The changes in the expression of cuticular proteins with cold shock are currently unexplained,
341 and we suggest that establishing how the cuticle structure is modified by cold shock, whether *Cpap3-d2*
342 is responsible for these changes, and how this contributes to overall cold tolerance maybe profitable
343 avenues for future research.

344

345 **5 Conclusion**

346

347 *Micrarchus* nov. sp. 2 regularly experiences sub-zero temperatures in the alpine environment. This
348 study is the first to apply high-throughput sequencing and *de novo* transcriptome alignment to

specifically address the response of a cold-hardy insect species to low temperature. This method clearly offers promise for future studies of cold tolerance in non-model organisms. In the current study RNA-Seq was a more successful approach than screening for *a priori* genes from other species. This allowed us to identify three *Micrarchus* nov. sp. 2 genes that responded to cold-shock, but had not been identified as cold-responsive in other insects.

Acknowledgments:. We would like to thank Jacob Corcoran for guidance with qPCR, Rich Leschen, Lucy Shield and Clive Williams for assistance with fieldwork, the Greymouth Department of Conservation office for access to Sewell Peak, Melody Clark for helpful comments on the manuscript and Katya Ruggiero for statistical support. The project was funded by The Royal Society of New Zealand Marsden Fund (LCR0901), the Allan Wilson Centre for Molecular Ecology and Evolution and Core funding for Crown Research Institutes from the Ministry of Business, Innovation and Employment's Science and Innovation Group.

References

Anders, S. and Huber, W. 2010. Differential expression analysis for sequence count data. Genome Biol. 11, R106.

Baker, D. and Russell, S. 2009. Gene expression during *Drosophila melanogaster* egg development before and after reproductive diapause. BMC Genomics 10, 242.

Bale, J.S. 2002.. Insects and low temperatures, from molecular biology to distributions and abundance. Philos. T. Roy. Soc. B 357, 849-862.

Batt, G.E. and Braun, J. 1999. The tectonic evolution of the Southern Alps, New Zealand, insights from fully thermally coupled dynamical modelling. Geophys. J. Int. 136, 403-420.

Batt, G.E., Braun, J., Kohn, B.P. and McDougall, I. 2000. Thermochronological analysis of the dynamics of the Southern Alps, New Zealand. Geol. Soc. Am. Bull. 112, 250-266.

Bilgen, T., English, T.E., McMullen, D.C. and Storey, K.B. 2001. *EsMlp*, a Muscle-LIM Protein Gene, Is Up-regulated during Cold Exposure in the Freeze-Avoiding Larvae of Epiblema scudderiana. Cryobiology 43, 11-20.

Buckley, T.R. and Simon, C. 2007. Evolutionary radiation of the cicada genus *Maoricicada* Dugdale Hemiptera, Cicadoidea. and the origins of the New Zealand alpine biota. Biol. J. Linn. Soc. 91, 419-435.

Buckley, T.R. and Bradler, S. 2010. *Tepakiphasma ngatikuri*, a new genus and species of stick insect Phasmatodea. from the Far North of New Zealand. N. Z. Entomol. 33, 118-126.

Carrasco, M.A., Buechler, S.A., Arnold, R.J., Sformo, T., Barnes, B.M. and Duman, J.G. 2011. Elucidating the Biochemical Overwintering Adaptations of Larval *Cucujus clavipes puniceus*, a Nonmodel Organism, via High Throughput Proteomics. J. Proteome Res. 10, 4634-4646.

Caudy, A.A., Ketting, R.F., Hammond, S.M., Denli, A.M., Bathoorn, A.M.P., Tops, B.B.J., Silva, J.M., Myers, M.M., Hannon, G.J. and Plasterk, R.H.A. 2003. A micrococcal nuclease homologue in RNAi effector complexes. Nature 425, 411-414.

Chamberlain, C.P. and Poage, M.A. 2000. Reconstructing the paleotopography of mountain belts from the isotopic composition of authigenic minerals. Geology 28, 115-118.

Chown, S.L. and Nicolson, S.W. 2004. Insect Physiological Ecology, Mechanisms and Patterns. Oxford University Press, Oxford.

Clark, M.S. and Worland, M. R. 2008. How insects survive the cold, molecular mechanisms-a review. J. Comp. Physiol. B 178, 917-933.

Colinet, H., Nguyen, T.T.A., Cloutier, C., Michaud, D. and Hance, T. 2007. Proteomic profiling of a parasitic wasp exposed to constant and fluctuating cold exposure. Insect Biochem. Mol. Biol. 37, 1177-1188.

Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M. and Robles, M. 2005. Blast2GO, a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674-3676.

Denlinger, D.L. and Lee, R.E. 2010. Low Temperature Biology of Insects. Cambridge, Cambridge University Press

Dittmer, N.T., Hiromasa, Y., Tomich, T.T., Lu, N. Beeman, R.W., Kramer, K.J and Kanost, M.R. 2011. Proteomic and transcriptomic analyses of rigid and membranous cuticles and epidermis from the elytra and hindwings of the red flour beetle, *Tribolium castaneum*. J. Proteome Res. 11, 269-278

Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Duran, C., Field, M., Heled, J., Kearse, M., Markowitz, S., Moir, R., Stones-Havas, S., Sturrock, S., Thierer, T. and Wilson, A. 2012. Geneious v5.6, available from http,//www.geneious.com/.

Gao, X., Ge, L., Shao, J., Su, C., Zhao, H., Saarikettu, J., Yao, X., Yao, Z., Silvennoinen, O. and Yang, J. 2010. Tudor-SN interacts with and co-localizes with G3BP in stress granules under stress conditions. FEBS Lett. 584, 3525-3532.

Goto, S.G. 2001.. A novel gene that is up-regulated during recovery from cold shock in *Drosophila melanogaster.* Gene 270, 259-264.

Gorres, K.L., Raines, R.T. 2010. Prolyl 4-hydoxylase. Crit. Rev. Biochem. Mol. Biol. 45, 106-124

Hardcastle, T. and Kelly, K. 2010. baySeq, Empirical Bayesian methods for identifying differential expression in sequence count data. BMC Bioinformatics 11, 422.

Hoffmann, A.A., Hallas, R., Anderson, A.R. and Telonis-Scott, M. 2005. Evidence for a robust sex-specific trade-off between cold resistance and starvation resistance in *Drosophila melanogaster*. J. Evol. Biol. 18, 804-810.

Hoffmann, A. A. 2010. Physiological climatic limits in Drosophila, patterns and implications. J. Exp. Biol. 213, 870-880.

Jasrapuria, S., Arakane, Y., Osman, G., Kramer, K.J., Beeman, R.W. and Muthukrishnan, S. 2010. Genes encoding proteins with peritrophin A-type chitin-binding domains in *Tribolium castaneum* are grouped into three distinct families based on phylogeny, expression and function. Insect Biochem. Molec. Biol. 40, 214-227.

Kallioniemi, E. and Hanski, I. 2011. Interactive effects of Pgi genotype and temperature on larval growth and survival in the Glanville fritillary butterfly. Funct. Ecol. 25, 1032-1039.

Karl, I., Schmitt, T. and Fischer, K. 2008. Phosphoglucose isomerase genotype affects life-history traits and cold stress resistance in a Copper butterfly. Funct. Ecol. 22, 887-894.

Kofler, R., Torres, T. T., Lelley, T. and Schlotterer, C. 2009. PanGEA, Identification of allele specific gene expression using the 454 technology. BMC Bioinformatics 10, 143.

Koštál, V., Renault, D., Mehrabianová, A. and Bastl, J. 2007.. Insect cold tolerance and repair of chill-injury at fluctuating thermal regimes, Role of ion homeostasis. Comp. Biochem. Phys. A 147, 231-238.

Laayouni, H, García-Franco, F, Chávez-Sandoval, B, Trotta, V, Beltran, S, Corominas, M and Santos, M 2007. Thermal evolution of gene expression profiles in *Drosophila subobscura*. BMC Evol. Biol. 7, 42.

Lee, R.E., Chen, C.-P. and Denlinger, D.L. 1987. A Rapid Cold-Hardening Process in Insects. Science 238, 1415-1417.

Li, A. and Denlinger, D.L. 2008. Rapid cold hardening elicits changes in brain protein profiles of the flesh fly, *Sarcophaga crassipalpis*. Insect Mol. Biol. 17, 565-572.

Mann, K., Mechling, D.E., Bächinger, H.P., Eckerskorn, C., Gaill, F. and Timpl, R. 1996. Glycosylated threonine but not 4-hydroxyproline dominates the triple helix stabilizing positions in the sequence of a hydrothermal vent worm cuticle collagen. J. Mol. Biol. 261, 255-266.

Michaud, M. and Denlinger, D.L. 2007. Shifts in the carbohydrate, polyol, and amino acid pools during rapid cold-hardening and diapause-associated cold-hardening in flesh flies *Sarcophaga crassipalpis*., a metabolomic comparison. J. Comp. Physiol. B 177, 753-763.

437 Overgaard, J., Sørensen, J.G., Petersen, S.O., Loeschcke, V. and Holmstrup, M. 2005.. Changes in membrane lipid
438 composition following rapid cold hardening in *Drosophila melanogaster*. J. Insect Physiol. 51, 1173-1182.
439 Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res.
440 29, 2002-2007
441 Purać, J., Burns, G., Thorne, M.A.S., Grubor-Lajšić, G., Worland, M.R. and Clark, M.S. 2008. Cold hardening
442 processes in the Antarctic springtail, *Cryptopygus antarcticus*, Clues from a microarray. J. Insect. Physiol.
443 54, 1356-1362.
444 Qin, W., Neal, S.J., Robertson, R.M., Westwood, J.T. and Walker, V.K. 2005. Cold hardening and transcriptional
445 change in *Drosophila melanogaster*. Insect Mol. Biol. 14, 607-613.
446 R Development Core Team 2012. R, A language and environment for statistical computing. Vienna, Austria, R
447 Foundation for Statistical Computing. Retrieved from http,//www.R-project.org.
448 Ramakers, C., Ruijter, J.M., Deprez, R.H.L. and Moorman, A.F.M. 2003. Assumption-free analysis of quantitative
449 real-time polymerase chain reaction PCR. data. Neurosci. Lett. 339, 62-66.
450 Rank, N.E., Bruce, D.A., McMillan, D.M., Barclay, C. and Dahlhoff, E.P. 2007. Phosphoglucose isomerase genotype
451 affects running speed and heat shock protein expression after exposure to extreme temperatures in a
452 montane willow beetle. J. Exp. Biol. 210, 750-764.
453 Rinehart, J.P., Li, A., Yocum, G.D., Robich, R.M., Hayward, S.A. and Denlinger, D.L. 2007. Up-regulation of heat
454 shock proteins is essential for cold survival during insect diapause. Proc. Natl. Acad. Sci. USA 104, 11130-
455 11137.
456 Robich, R.M., Rinehart, J.P., Kitchen, L.J. and Denlinger, D.L. 2007. Diapause-specific gene expression in the
457 northern house mosquito, *Culex pipiens* L., identified by suppressive subtractive hybridization. J. Insect
458 Physiol. 53, 235–245.
459 Robinson, M.D., McCarthy, D.J. and Smyth, G.K. 2010. edgeR, a Bioconductor package for differential expression
460 analysis of digital gene expression data. Bioinformatics 26, 139-140.
461 Rowland, L.J., Dhanaraj, A.L., Naik, D., Alkharouf, N., Matthews, B. and Arora, R. 2008. Study of Cold Tolerance in
462 Blueberry Using EST Libraries, cDNA Microarrays, and Subtractive Hybridization. HortSci. 43, 1975-1981.
463 Rozen, S. and Helen, J. 2000. Primer3 on the WWW for general users and for biologist programmers. In, Krawetz S,
464 Misener S eds. Bioinformatics Methods and Protocols, Methods in Molecular Biology. Humana Press,
465 Totowa, NJ, pp 365-386.
466 Salmon, J.T. 1991. The Stick Insects of New Zealand. Reed, Auckland, New Zealand
467 Shoulders, M.D. and Raines, R.T. 2009. Collagen Structure and Stability. Annu. Rev. Biochem. 78, 929-958.
468 Sinclair, B.J., Worland, M.R., and Wharton, D.A. 1999. Ice nucleation and freezing tolerance in New Zealand alpine
469 and lowland weta, *Hemideina* spp. Orthoptera; Stenopelmatidae.. Physiol. Entomol. 24, 56-63.
470 Sinclair, B.J., Vernon, P., Klok, C.J. and Chown, S.L. 2003. Insects at low temperatures, an ecological perspective.
471 Trends Ecol. Evol. 18, 257-262.
472 Sinclair, B.J., Gibbs, A.G. and Roberts, S.P. 2007. Gene transcription during exposure to, and recovery from, cold
473 and desiccation stress in *Drosophila melanogaster*. Insect Mol. Biol. 16, 435-443.
474 Sørensen, J.G., Nielsen, M.M. and Loeschcke, V. 2007. Gene expression profile analysis of *Drosophila*
475 *melanogaster* selected for resistance to environmental stressors. J. Evol. Biol. 20, 1624-1636.
476 Storey, K.B. and Storey, J.M. 2012. Insect cold hardiness, metabolic, gene, and protein adaptation. Can. J. Zool. 90,
477 456-475
478 Sundstrom, J.F., Vaculova, A., Smertenko, A.P., Savenkov, E.I., Golovko, A., Minina, E., Tiwari, B.S., Rodriguez-
479 Nieto, S., Zamyatnin, A.A., Valineva, T., Saarikettu, J., Frilander, M.J., Suarez, M.F., Zavialov, A., Stahl, U.,
480 Hussey, P.J., Silvennoinen, O., Sundberg, E., Zhivotovsky, B. and Bozhkov, P.V. 2009. Tudor staphylococcal
481 nuclease is an evolutionarily conserved component of the programmed cell death degradome. Nature Cell
482 Biol. 11, 1347-1354.

Teets, N.M., Peyton, J.T., Ragland, G.J., Colinet, H., Renault, D., Hahn, D.A. and Denlinger, D.L. 2012. Uncovering molecular mechanisms of cold tolerance in a temperate flesh fly using a combined transcriptomic and metabolomic approach. Physiol. Genomics 44, 764-2012

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, research0034

Vonk, L.A., Doulabi, B.Z., Huang, C.-L., Helder, M.N., Everts, V. and Bank, R.A. 2010. Endoplasmic reticulum stress inhibits collagen synthesis independent of collagen-modifying enzymes in different chondrocyte populations and dermal fibroblasts. Biochem Cell. Biol. 88, 539-552.

Wang, Z., Gerstein, M. and Snyder, M. 2009. RNA-Seq, a revolutionary tool for transcriptomics. Nature Rev. Genet. 10, 57-63.

Weissbach, R. and Scadden, A.D.J. 2012. Tudor-SN and ADAR1 are components of cytoplasmic stress granules. RNA 18, 462-471.

Wharton, D.A. 2011. Cold tolerance of New Zealand alpine insects. J. Insect Physiol. 57, 1090-1095.

Yan, S.P., Zhang, Q.Y., Tang, Z.C., Su, W.A. and Sun, W.N. 2006. Comparative Proteomic Analysis Provides New Insights into Chilling Stress Responses in Rice. Mol. Cell Proteomics 5, 484-496.

Yi, S.X., Moore, C.W. and Lee, R.E. 2007. Rapid cold-hardening protects *Drosophila melanogaster* from cold-induced apoptosis. Apoptosis 12, 1183-93.

Ying, M. and Chen, D. 2012. Tudor domain-containing proteins of *Drosophila melanogaster*. Develop. Growth Differ. 54, 32-43

Zhang, J., Marshall, K.E., Westwood, J.T., Clark, M.S. and Sinclair, B.J. 2011. Divergent transcriptomic responses to repeated and single cold exposures in *Drosophila melanogaster*. J. Exp. Biol. 214, 4021-4029.

Zieger, M., Gupta, M. and Wang, M. 2011. Proteomic analysis of endothelial cold-adaptation. BMC Genomics 12, 630.

Figures

Figure 1: Species distribution of the top BLASTx matches for each annotated contig from the *de* novo assembled *Micrarchus* nov. sp. 2 transcriptome. Out of the 5,235 contigs, 2,060 (39 %) matched entries in the non redundant protein (nr) database using Blast2GO (E-value cut-off < 1^{-10}).

Figure 2: Volcano plot showing relative mRNA abundances of each contig in the *Micrarchus* nov. sp. 2 transcriptome between non cold-treated Individuals and those exposed to 0 ^{o}C for one hour, with a further hour recovery (n = 2). Differential expression analysis was performed with an exact test approach using edgeR with common dispersion. Grey points = contigs with P-value > 0.05, black points = contigs with P-value <0.05. Shaded boxes represent areas with a significant P-value and at least a two-fold change in mRNA abundance as a result of treatment. Contigs where expression is not recorded in one of the treatments, resulting in an infinite fold change, are not plotted. The three contigs verified as differentially expressed using qPCR are labelled: prolyl 4-hydroxylase subunit alpha-1 (*P4HA1*), staphylococcal nuclease domain-containing protein 1 (*snd1*) and a cuticular protein analogous to peritrophins 3-D2 (*Cpap3-d2*).

524

525 **Figure 3:** Boxplots showing relative mRNA abundance from qPCR for each gene between non cold-
526 treated (white box plots) *Micrarchus* nov. sp. 2 individuals and those exposed to 0 ^{o}C for one hour, with
527 a further hour recovery (grey box plot) (*n* = 3). Boxplot pairs on a grey background represent the
528 reference genes used for normalisation of abundances between treatments. Boxplots on white
529 background in top panel depict differentially expressed genes identified from RNA-Seq data (**Table 2**).
530 Boxplots on white background in bottom panel shows *a priori* genes identified in previous studies
531 (**Table 3**). * = samples with significant t-test *P*-values (< 0.05) from natural log transformed relative
532 abundance values. Dark line indicates median, box represents 25th and 75th percentile with whiskers
533 representing maximum and minimum values.
534

535 **Table 1:** The number of raw 454 sequences obtained for each *Micrarchus* nov. sp. 2 cDNA library, its
536 treatment, and the percentage used in the final *de novo* transcriptome alignment. Raw data was
537 submitted to the National Centre for Biotechnology and Information (NCBI) with Sequence Read
538 Archive (SRA) submission number **SRA057228**.

539

Sample	Treatment	# Sequences	# Sequences in alignment
MMT016	Cold-shock	101,379	79,382 (78.3%)
MMT017	Cold-shock	107,242	81,039 (75.6%)
MMT018	Control	160,584	124,300 (77.4%)
MMT019	Control	154,915	118,845 (76.8%)
MMT086	Control	44,480	68,779 (82.8%)
MMT0135	Cold-shock	38,632	
	Total:	607,232	472345 (77.8%)

540

541

Table 2: Differential expression analysis results for the top ranked up-regulated genes in response to cold-shock from *Micrarchus* nov. sp. 2 RNA-Seq data. The relative mRNA abundances between individuals exposed to 0 ^{o}C for one hour, with a further hour recovery, were compared to non cold-treated individuals (*n* = 2). Contigs were ranked by *P*-value/Likelihood depending on which analysis was used, boxes shaded grey are non-significant results. Three exact test approaches (edgeR with common dispersion, edgeR with tagwise dispersion and DESeq) and two Bayesian methods (baySeq with and without contig length) were performed. All top four ranked contigs for each analysis shown were selected for validation using qPCR (n = 11 due to redundancy between results).

Contig	Results	edgeR (common dispersion)	edgeR (tagwise dispersion)	DESeq	baySeq (without length)	baySeq (with length)
snd1	Rank	22	1	1	859	2
	Result	4.98E-03	3.08E-04	5.37E-03	0.066	0.816
C-01744	Rank	37	30	29	3**	33
	Result	1.68E-02	1.87E-02	1.53E-01	0.715	0.517
Cpap3-d2	Rank	46	4	21	1	4**
	Result	1.90E-02	3.88E-03	1.10E-01	0.815	0.774
P4HA1	Rank	47	7	24	2	1
	Result	2.19E-02	5.12E-03	1.23E-01	0.803	0.871
Copia	Rank	9	23	4	28	47
	Result	2.47E-03	1.43E-02	3.65E-02	0.463	0.617
*cadn**	Rank	1	2	2	186	77
	Result	1.72E-04	7.79E-04	6.49E-03	0.207	0.352
csde1	Rank	21	3	8	10	6
	Result	4.74E-03	3.48E-03	5.22E-02	0.602	0.729
C-04525	Rank	29	22	20	4**	3**
	Result	7.94E-03	1.27E-02	1.03E-01	0.709	0.780
SF3a	Rank	2	5	3	551	55
	Result	5.12E-04	3.88E-03	1.57E-02	0.109	0.422
restin	Rank	3	11	7	515	37
	Result	2.52E-02	8.73E-03	4.50E-02	0.115	0.485
*thil**	Rank	4	6	6	95	23
	Result	1.80E-03	4.88E-03	4.46E-02	0.295	0.585

Full contig BLAST annotations are: *snd1* = staphylococcal nuclease domain-containing protein 1, *C-01744* = non annotated contig 01744, *Cpap3-d2* = Cuticular protein analogous to peritrophins 3-D3, *P4HA1* = Prolyl 4-hydroxylase subunit alpha-1, *Copia* = Copia protein, *cadn* = neural cadherin, *csde1* = cold shock domain-containing protein e1, *C-04525* = non annotated contig 04525, *SF3a* = Spliceosome associated protein and *restin* = restin. Rank of contig out of: 1,186 for edgeR with common dispersion, 1,188 for edgeR with tagwise dispersion, 1,292 for DESeq and 1,145 for both baySeq analyses. Results show *P*-value for edgeR & DESeq, likelihood for baySeq. * = contig not used in subsequent qPCR analysis due to non-specific amplification of target DNA. ** = in the top four up-regulated genes for baySeq analyses, but the robust approach means the likelihood value is non-significant.

560 **Table 3**: The eight *a priori* candidate genes for qPCR analysis present in the *Micrarchus* nov. sp. 2
561 transcriptome that have been previously identified in low temperature studies as having a role in cold
562 tolerance. All genes/proteins were shown to be up-regulated in response to low temperature except for
563 *glna2* (gene product up-regulated) and *pgi* (genotype, not regulation, affects cold stress resistance).

Gene		Species
atpa	ATP synthase subunit alpha	*Sarcophaga crassipalpis* (Li and Denlinger, 2008)
glna2	glutamine synthetase 2	*S. crassipalpis* (Michaud and Denlinger, 2007)
hsp70	heat shock protein 70	*Aphidius colemani* (Colinet *et al.,* 2007)
hsp90	heat shock protein 90	*Drosophila melanogaster* (Qin *et al*., 2005)
NDUFA	NADH: ubiquinone dehydrogenase	*Oryza sativa** (Yan *et al*., 2006)
pgi	phosphoglucose isomerase	*Melitaea cinxia* (Kallioniemi and Hanski, 2011)*, Lycaena tityrus* (Karl *et al.,* 2008)*, Chrysomela aeneicollis* (Rank *et al.,* 2007)
dcam	S-adenosylmethionine decarboxylase proenzyme	*Vaccinium corymbosum** (Rowland *et al.,* 2008)
sahha	S-adenosyl-l-homocysteine hydrolase a	*Homo sapiens** (Zieger *et al.,* 2011)

564

565 * = are genes from species that are not insects. These genes were selected for qPCR analysis as they were also in the top 10 %
566 of up-regulated contigs in the RNA-Seq data (ranked by *P*-value from the edgeR with tagwise dispersion)

567

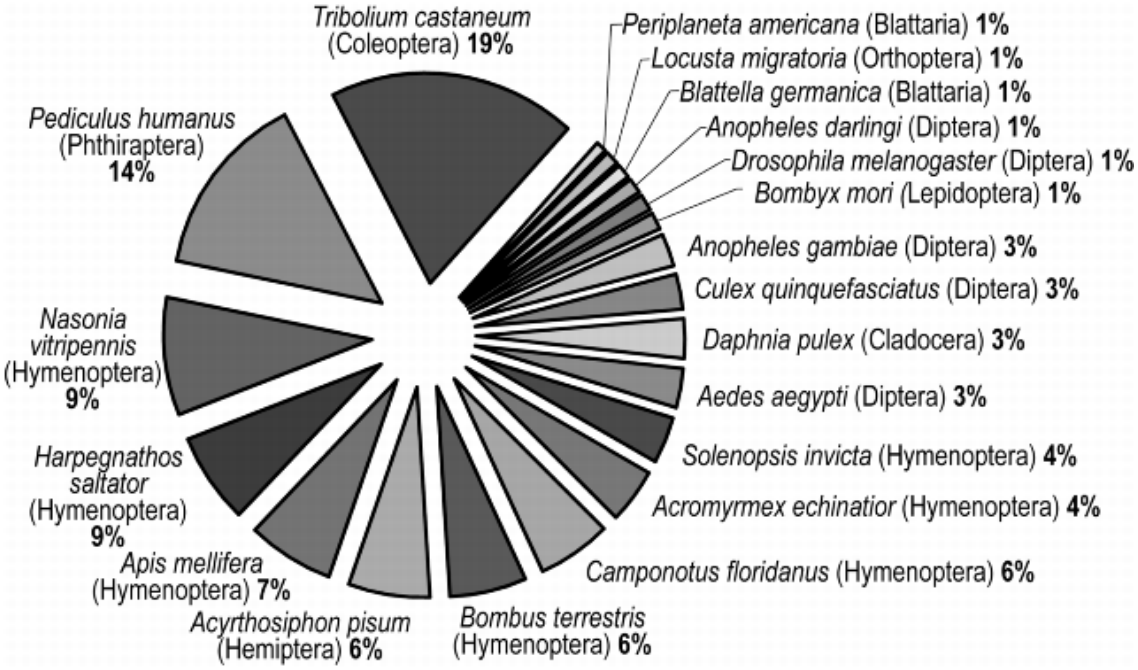
Tribolium castaneum (Coleoptera) 19%
Periplaneta americana (Blattaria) 1%
Locusta migratoria (Orthoptera) 1%
Blattella germanica (Blattaria) 1%
Anopheles darlingi (Diptera) 1%
Drosophila melanogaster (Diptera) 1%
Bombyx mori (Lepidoptera) 1%
Anopheles gambiae (Diptera) 3%
Culex quinquefasciatus (Diptera) 3%
Daphnia pulex (Cladocera) 3%
Aedes aegypti (Diptera) 3%
Solenopsis invicta (Hymenoptera) 4%
Acromyrmex echinatior (Hymenoptera) 4%
Camponotus floridanus (Hymenoptera) 6%
Bombus terrestris (Hymenoptera) 6%
Acyrthosiphon pisum (Hemiptera) 6%
Apis mellifera (Hymenoptera) 7%
Harpegnathos saltator (Hymenoptera) 9%
Nasonia vitripennis (Hymenoptera) 9%
Pediculus humanus (Phthiraptera) 14%

Figure 1

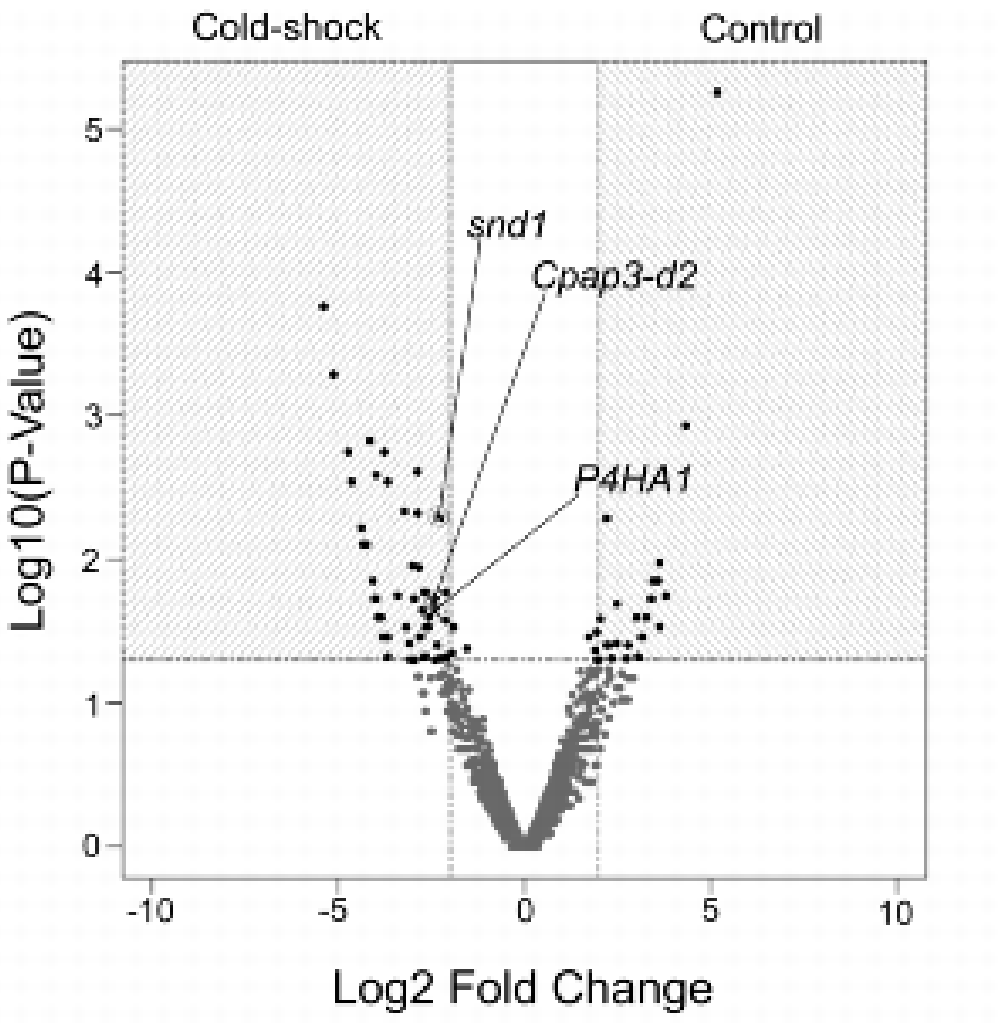
Cold-shock
Control
snd1
Cpap3-d2
P4HA1
Log10(P-Value)
Log2 Fold Change
0
1
2
3
4
5
-10
-5
0
5
10

Figure 2

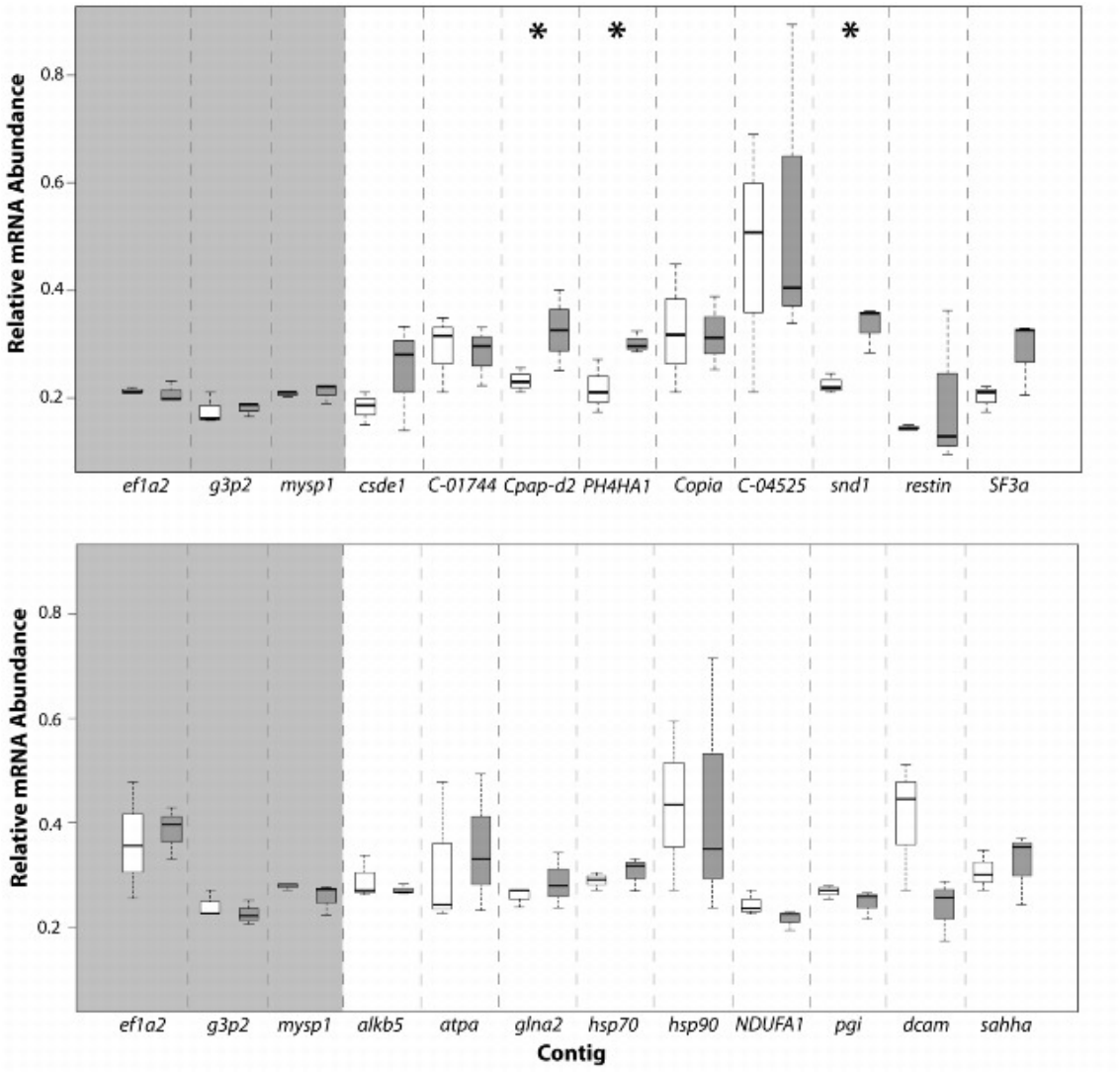
Relative mRNA Abundance
0.8
0.6
0.4
0.2
*
*
*
ef1a2
g3p2
mysp1
csde1
C-01744
Cpap-d2
PH4HA1
Copia
C-04525
snd1
restin
SF3a
Relative mRNA Abundance
0.8
0.6
0.4
0.2
ef1a2
g3p2
mysp1
alkb5
atpa
glna2
hsp70
hsp90
NDUFA1
pgi
dcam
sahha
Contig

Figure 3

Supplementary material A -Primer sequences for RT-qPCR. Annotations from BLASTx against SwissProt database unless otherwise stated.

Annotation (e-value < 1^{-10})	Primer	Sequence (5' - 3')
atp synthase subunit alpha	atpa_F1	TCGAGCGCGAGTTCCTCCAA
(*atpa*)	atpa_R1	TTGCCCTCCTTCGCGATGCT
Paramyosin long form	mysp1_F1	TGCTTGGCGCGGATAAGCGA
(*mysp1*)	mysp1_R1	AGGCAGCTACAAGAACAGGAGGGC
glutamine synthetase 2 cytoplasmic	glna2_F1	TCTTCGCGCATCGCCTTGGT
(*glna2*)	glna2_R1	TCGTGGTGACCTTCGACCCCAA
s-adenosylmethionine decarboxylase proenzyme	dcam_F1	CACGTTTGACCTGTTCATACCAGCG
(*dcam*)	dcam_R1	ACCAATGAGAGGGACAGCAAGAACA
heat shock protein 90	hsp90_F1	CATGCGGCCAGGATATACCGCA
(*hsp90*)	hsp90 _R1	AGCATCCTCGCCTTCCGCTT
staphylococcal nuclease domain-containing protein 1	snd1_F1	TCGCAAAAGCTCGCACCGCT
(*snd1*)	snd1_R1	TGTGCCTCTCTCCCTTCAGGCT
heat shock protein 70	hsp70_F1	GGGGATGGTGGTGTTGCGCTTT
(*hsp70*)	hsp70_R1	GCCATCTTGGCTGGCGACAAGT
elongation factor 1-alpha 2	ef1a2_F1	TCTCTGCCCTGCCGACTGTCAT
(*ef1a2*)	ef1a2_R1	ACCGGCCTTTCCTGCGAAACT
Unannotated contig01744	C-01744_F1	TGCGAAGACAAGCTTTAGAGACCGT
(*C-01744*)	C-01744_R1	ACCCTCTTAAGGACGAGCGCCA
s-adenosyl-l-homocysteine hydrolase a	sahha_F1	GCTGCCTGCAGCGCATTTATGG
(*sahha*)	sahha_R1	TGTTGGCGGGAAAGGTGGCA
glyceraldehyde-3-phosphate dehydrogenase 2	g3p2_F1	ACCGTCCCTCCACAACTTGCCA
(*g3p2*)	g3p2_R1	TGGCACCACTTGCCAAGGTCA
Unannotated contig01822	C-01822_F1	AGGTGAAGCCGAAAGGTTGGCA
(*C-01822*)	C-01822_R1	AGGACTGCGAAATCGTCCTCC
Phosphoglucose isomerase	pgi_F1	ACTACGCCACAGGCCCCATAGT
(*pgi*)	pgi_R1	TGGTGGATCAGCTGGTAGAAGGC
Unannotated contig02740	C-02740_F1	AGATGGTTACACATGCCACACCCAA
(*C-02740*)	C-02740_R1	ACAGGCAAGGGTGTGTTGCCA
Unannotated contig02855	C-02855_F1	AGCCTTGACACCACTCCTTCCAAC
(*C-02855*)	C-02855_R1	TGAAGACTGCGGGTGAGCTGT
probable alpha-ketoglutarate-dependent dioxygenase	alkb5_F1	TCGTTTCGACAGTTGCGACCCA
(*alkb5*)	alkb5_R1	TGACCGGGCCCCTCTTAGAAACA

cold shock domain-containing protein e1 (*csde1*)	csde1_F1	TTGTCGCCAGAACGCAGGGT
	csde1_R1	AACCGCGGGGAGTGCTTCTT
Unannotated contig04525 (*C-04525*)	C-04525_F1	TGCTTTGGAGGCGGGCTTGT
	C-04525_R1	CGGCAACAGCGAAGTGCTGA
nadh: ubiquinone dehydrogenase (BLAST x / *nr*) (*NDUFA1*)	NDUFA1_F1	TGTCTTGGGAGTTGCTTTGAAGTGG
	NDUFA1_R1	GGACACGGTGGTACTGTGTTTGACA
Tribolium castaneum spliceosome associated protein mrna (BLAST n / *nr*) (*SF3a*)	SF3a_F1	GGCTGGCCAAAGAAGCCAAGGA
	SF3a_R1	TTGCTGCAGTTGGCGTGGCT
restin (*restin*)	restin_F1	TCCACCAGCCTCTGCAAGTCCT
	restin_R1	TGACGCGTGCTGCCAGTCAA

Supplementary material - File B – Contigs from Micrarchus nov. sp. 2 transcriptome used for qPCR analysis

	Selected[1]	Contig Length (bp)	BLASTx against nr database	E-Value	BLASTn against nr database	E-Value	BLASTx against SwissProt database	E-Value	MMT16 (cold-shock) Counts[2]	MMT17 (cold-shock) Counts[2]	MMT18 (control) Counts[2]	MMT19 (control) Counts[2]	qPCR one-tailed t-test *P*-value
P4HA1	DE	755	Prolyl 4-hydroxylase subunit alpha-1 [Acromyrmex echinatior]	3.21E-120	PREDICTED: Nasonia vitripennis prolyl 4-hydroxylase subunit alpha-2-like (LOC100116138), mRNA	3.45E-105	Prolyl 4-hydroxylase subunit alpha-1; Short=4-PH alpha-1; AltName: Full=Procollagen-proline,2-oxoglutarate-4-dioxygenase subunit alpha-1	5.78E-91	17	14	2	3	3.27E-02
snd1	DE	2383	ebna2 binding protein p100	0.0E+00	strongylocentrotus purpuratus staphylococcal nuclease domain containing 1 mrna	1.5E-61	snd1_ponab ame: full=staphylococcal nuclease domain-containing protein 1 ame: full=100 kda coactivator ame: full=p100 co-activator	0.0E+00	93	33	12	13	6.51E-03
C-04525	DE	441	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	9	8	1	0	3.65E-01
Cpap3-d2	DE	1333	Nasonia vitripennis cuticular protein analogous to peritrophins 3-D2 (Cpap3-d2), mRNA	1.13E-160	cuticular protein analogous to peritrophins 3-D2 precursor [Tribolium castaneum]	6.68E-120	---NA---	---NA---	17	15	3	2	4.69E-02
csde1	DE	568	cold shock domain-containing protein e1-like isoform 3	1.2E-36	acyrthosiphon pisum cold shock domain-containing protein e1- transcript variant 3 mrna	1.9E-65	csde1_human ame: full=cold shock domain-containing protein e1 ame: full=n-ras upstream gene protein ame: full=protein unr	2.1E-11	30	7	3	2	2.01E-01
Copia	DE	724	hypothetical protein TcasGA2_TC015470 [Tribolium castaneum]	4.34E-72	Medicago truncatula chromosome 8 clone mth2-182o15, complete sequence	4.48E-12	Copia protein; AltName: Full=Gag-int-pol protein; Contains: RecName: Full=Copia VLP protein; Contains: RecName: Full=Copia protease	1.57E-38	43	0	3	2	4.91E-01
C-01744	DE	1461	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	11	10	3	1	4.69E-01
restin	DE	285	restin (reed-steinberg cell-expressed intermediate filament-associated protein)	3.3E-18	---NA---	---NA---	---NA---	---NA---	30	0	2	0	3.83E-01
SF3a	DE	357	---NA---	---NA---	tribolium castaneum spliceosome associated protein mrna	5.2E-13	---NA---	---NA---	0	28	0	1	6.15E-02
cadn	DE	637	cadherin- isoform h	1.7E-39	acyrthosiphon pisum neural-cadherin-like mrna	9.7E-51	cadn_drome ame: full=neural-cadherin ame: full=cadherin-n short=dn-cadherin flags: precursor	2.0E-31	2	33	1	0	NA*
thil							thil_mouse ame: full=acetyl-						

	DE	328	acetoacetyl- thiolase	2.1E-27	tetraodon nigroviridis full-length cdna	8.6E-29	mitochondrial ame: full=acetoacetyl- thiolase flags: precursor	2.5E-25	4	18	0	1	NA*
atpa	*a priori*	1938	atp synthase subunit mitochondrial	0.0E+00	drosophila erecta gg22793 (dere\gg22793) mrna	0.0E+00	atpa_drome ame: full=atp synthase subunit mitochondrial ame: full=protein bellwether flags: precursor	0.0E+00	66	61	76	61	3.63E-01
glna2	*a priori*	4702	glutamine synthetase 2 cytoplasmic	5.7E-159	drosophila erecta gg18416 (dere\gg18416) mrna	2.0E-158	glna2_drome ame: full=glutamine synthetase 2 cytoplasmic ame: full=glutamate--ammonia ligase 2	2.4E-149	70	85	48	48	2.46E-01
dcam	*a priori*	3811	s-adenosylmethionine decarboxylase proenzyme	4.4E-144	pediculus humanus corporis s-adenosylmethionine mrna	7.4E-49	dcam_drome ame: full=s-adenosylmethionine decarboxylase proenzyme short= etdc short=samdc contains: ame: full=s-adenosylmethionine decarboxylase alpha chain contains: ame: full=s-adenosylmethionine decarboxylase beta chain flags: precursor	1.1E-104	121	140	16	72	5.02E-02
hsp70	*a priori*	2264	heat shock protein 70	0.0E+00	drosophila melanogaster heat shock protein cognate 4 (hsc70-4) transcript variant mrna	0.0E+00	hsp7d_manse ame: full=heat shock 70 kda protein cognate 4 short=hsc 70-4	0.0E+00	253	294	308	258	2.48E-01
sahha	*a priori*	1453	s-adenosylhomocysteine hydrolase	0.0E+00	tetraodon nigroviridis full-length cdna	0.0E+00	sahha_xenla ame: full=adenosylhomocysteinase a short= cyase a ame: full=s-adenosyl-l-homocysteine hydrolase a	0.0E+00	27	33	15	10	4.02E-01
pgi	*a priori*	902	phosphoglucose isomerase	2.4E-175	drosophila sechellia gm20655 (dsec\gm20655) mrna	1.7E-163	g6pi_droya ame: full=glucose-6-phosphate isomerase short=gpi ame: full=phosphoglucose isomerase short=pgi ame: full=phosphohexose isomerase short=phi	1.7E-168	3	7	2	4	1.47E-01
NDUFA1	*a priori*	428	nadh:ubiquinone dehydrogenase	1.4E-33	---NA---	---NA---	---NA---	---NA---	13	7	3	1	9.65E-02
hsp90	*a priori*	2608	heat shock protein 90	0.0E+00	locusta migratoria heat shock protein 90 complete cds	0.0E+00	hs90b_horse ame: full=heat shock protein hsp 90-beta	0.0E+00	62	47	43	43	4.47E-01
alkb5	Counts	704	probable alpha-ketoglutarate-dependent dioxygenase abh5	7.9E-55	xenopus laevis alkylation repair homolog 5 mrna	9.5E-39	alkb5_xenla ame: full=probable alpha-ketoglutarate-dependent dioxygenase abh5 ame: full=alkylated dna repair protein alkb homolog 5	1.4E-53	8	9	2	1	2.50E-01
mysp1	Reference	3708	long form-like	0.0E+00	nasonia vitripennis long	0.0E+00	mysp1_drome ame: full=	0.0E+00	1275	1142	1477	1074	NA

					form-like mrna		long form						
efia2	Reference	1989	elongation factor 1-alpha	0.0E+00	drosophila melanogaster elongation factor 1alpha48d transcript variant mrna	0.0E+00	ef1a2_drome ame: full=elongation factor 1-alpha 2 short=ef-1-alpha-2	0.0E+00	327	333	350	369	NA
g3p2	Reference	1433	glyceraldehyde-3-phosphate dehydrogenase	0.0E+00	oncometopia nigricans glyceraldehyde-3-phosphate dehydrogenase complete cds	0.0E+00	g3p2_drome ame: full=glyceraldehyde-3-phosphate dehydrogenase 2 ame: full=glyceraldehyde-3-phosphate dehydrogenase ii short=gapdh ii	0.0E+00	86	70	87	83	NA

Selected[1] = Selected for qPCR from the differential expression analysis (DE), from the literature (*a priori*) or the by eye from the raw counts (Counts)

Counts[2] = edgeR quantile corrected counts used for differential expression analysis

[*] = not use for qPCR analysis due to non-specific amplification of target DNA based on melt curve s

Supplementary File C - Significant expression results

Contig	Length (	Blastx (*nr*)	*E*-value	Blastn (*nr*)	*E*-value	Blastx (SwissProt)	*E*-value	quantile normalised edgeR counts MMT16	MMT17	MMT18	MMT19	Regulation	Common Dispersion edgeR *P*-value	Tagwise Dispersion edgeR *P*-value	DESeq *P*-value	baySeq (Likelihood)	including contig length baySeq (Likelihood)
isotig01686	1583	antibacterial peptide	2.5E-17	---NA---	---NA---	---NA---	---NA---	0	0	0	55	Downregulated	1.08E-06	2.36E-05	6.14E-03	non-signifincat	non-signifincat
isotig01320_isotig013	693	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	2	1	85	Downregulated	5.52E-06	4.76E-05	1.47E-02	non-signifincat	non-signifincat
isotig03225	637	cadherin- isoform h	1.7E-39	acyrthosiphon pisum	9.7E-51	cadn_drome ame: full	2.0E-31	2	33	1	0	Upregulated	1.72E-04	7.79E-04	6.49E-03	non-signifincat	non-signifincat
isotig02018	1098	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	8	16	Downregulated	1.98E-04	2.33E-04	non-signifincat	non-signifincat	non-signifincat
isotig05226	357	---NA---	---NA---	tribolium castaneum s	5.2E-13	---NA---	---NA---	0	28	0	1	Upregulated	5.12E-04	3.88E-03	1.57E-02	non-signifincat	non-signifincat
isotig02239	956	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	6	14	Downregulated	5.24E-04	1.02E-03	non-signifincat	non-signifincat	non-signifincat
isotig01954	1159	cral trio domain-containing protein	5.1E-42	drosophila mojavensis	3.5E-110	---NA---	---NA---	0	0	14	3	Downregulated	8.92E-04	2.72E-03	non-signifincat	non-signifincat	non-signifincat
isotig01929	1190	protein phosphatase 1 regulatory sul	8.3E-50	anopheles gambiae p	4.4E-52	---NA---	---NA---	0	1	9	15	Downregulated	1.16E-03	1.10E-03	non-signifincat	non-signifincat	non-signifincat
isotig05565	285	restin (reed-steinberg cell-expressed	3.3E-18	---NA---	---NA---	---NA---	---NA---	30	0	2	0	Upregulated	1.47E-03	8.73E-03	4.50E-02	non-signifincat	non-signifincat
isotig01081_isotig010	528	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	0	16	Downregulated	1.58E-03	1.17E-02	non-signifincat	non-signifincat	non-signifincat
isotig05386	328	acetoacetyl- thiolase	2.1E-27	tetraodon nigroviridis	8.6E-29	thil_mouse ame: full=	2.5E-25	4	18	0	1	Upregulated	1.80E-03	4.88E-03	4.46E-02	non-signifincat	non-signifincat
isotig02147	1001	neurogenic locus notch-like protein p	4.6E-167	apis mellifera hypothe	1.7E-75	notc3_human ame: fu	1.2E-22	20	2	1	1	Upregulated	1.80E-03	6.04E-03	non-signifincat	non-signifincat	non-signifincat
isotig05504	306	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	1	14	0	0	Upregulated	2.13E-03	1.20E-02	non-signifincat	non-signifincat	non-signifincat
isotig05614	256	hydroxypyruvate isomerase	1.9E-19	bombus terrestris hyd	5.3E-11	ygbm_ecoli ame: full=	2.0E-13	1	14	0	0	Upregulated	2.13E-03	1.20E-02	non-signifincat	non-signifincat	non-signifincat
isotig05454	317	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	1	14	0	0	Upregulated	2.13E-03	1.22E-02	non-signifincat	non-signifincat	non-signifincat
isotig02855	724	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	43	0	3	2	Upregulated	2.47E-03	1.43E-02	3.65E-02	non-signifincat	non-signifincat
isotig05676	192	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	26	0	2	0	Upregulated	2.57E-03	1.66E-02	non-signifincat	non-signifincat	non-signifincat
isotig05606	261	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	2	19	1	1	Upregulated	2.87E-03	8.88E-03	non-signifincat	non-signifincat	non-signifincat
isotig03297	625	cytochrome p450 6a1	1.3E-25	---NA---	---NA---	cp6j1_blage ame: full	1.2E-19	0	20	0	1	Upregulated	2.87E-03	1.83E-02	non-signifincat	non-signifincat	non-signifincat
isotig03759	548	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	8	7	0	0	Upregulated	2.91E-03	9.18E-03	non-signifincat	non-signifincat	non-signifincat
isotig05738	122	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	8	7	0	0	Upregulated	2.91E-03	9.18E-03	non-signifincat	non-signifincat	non-signifincat
isotig02398	882	camp-responsive element-binding 2-	1.1E-44	tribolium castaneum c	3.9E-32	crbl2_mouse ame: ful	6.3E-28	0	0	4	10	Downregulated	2.91E-03	7.44E-03	non-signifincat	non-signifincat	non-signifincat
isotig04682	427	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	10	5	0	0	Upregulated	2.91E-03	1.01E-02	non-signifincat	non-signifincat	non-signifincat
isotig02379	891	basic chitinase 2-2	1.5E-130	musa ab group chitina	8.7E-123	chia_secce ame: full=	8.4E-125	0	0	3	10	Downregulated	2.91E-03	1.20E-02	non-signifincat	non-signifincat	non-signifincat
isotig04455	449	viral a-type inclusion protein repeat	1.5E-11	---NA---	---NA---	---NA---	---NA---	12	2	0	0	Upregulated	2.91E-03	1.07E-02	non-signifincat	non-signifincat	non-signifincat
isotig00367_isotig003	628	beta 2c-like	1.2E-113	acyrthosiphon pisum	1.5E-130	tbb1_manse ame: full	5.0E-114	1	12	0	0	Upregulated	2.91E-03	1.68E-02	non-signifincat	non-signifincat	non-signifincat
isotig04239	472	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	9	5	0	0	Upregulated	4.04E-03	1.56E-02	non-signifincat	non-signifincat	non-signifincat
isotig02044	1069	zinc finger bed domain-containing pr	1.7E-26	---NA---	---NA---	---NA---	---NA---	0	0	2	10	Downregulated	4.04E-03	1.44E-02	non-signifincat	non-signifincat	non-signifincat
isotig05372	339	neural wiskott-aldrich syndrome	2.6E-14	anopheles gambiae p	2.1E-11	wasp_human ame: fu	2.8E-12	0	14	0	0	Upregulated	4.04E-03	2.56E-02	non-signifincat	non-signifincat	non-signifincat
isotig04258	475	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	20	4	1	2	Upregulated	4.68E-03	9.84E-03	non-signifincat	non-signifincat	non-signifincat
isotig03626	568	cold shock domain-containing protei	1.2E-36	acyrthosiphon pisum	1.9E-65	csde1_human ame: fu	2.1E-11	30	7	3	2	Upregulated	4.74E-03	3.48E-03	non-signifincat	non-signifincat	non-signifincat
isotig01497	2383	ebna2 binding protein p100	0.0E+00	strongylocentrotus pu	1.5E-61	snd1_ponab ame: full	0.0E+00	93	33	12	13	Upregulated	4.98E-03	3.08E-04	5.37E-03	non-signifincat	non-signifincat
isotig01284_isotig012	1045	dfp_locmi ame: full= defense protein	3.2E-25	---NA---	---NA---	dfp_locmi ame: full= c	1.0E-26	24	58	37	338	Downregulated	5.17E-03	3.61E-03	3.96E-02	non-signifincat	non-signifincat
isotig04672	424	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	2	9	Downregulated	5.66E-03	1.97E-02	non-signifincat	non-signifincat	non-signifincat
isotig04478	447	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	10	2	0	0	Upregulated	5.66E-03	2.65E-02	non-signifincat	non-signifincat	non-signifincat
isotig05246	354	rab-like protein 3	2.2E-51	aedes aegypti small g	6.3E-31	rabl3_chick ame: full=	8.0E-41	10	2	0	0	Upregulated	5.66E-03	2.65E-02	non-signifincat	non-signifincat	non-signifincat
isotig05509	304	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	1	11	Downregulated	5.66E-03	2.72E-02	non-signifincat	non-signifincat	non-signifincat
isotig04177	485	PREDICTED: hypothetical protein L(	4.6E-13	---NA---	---NA---	---NA---	---NA---	11	1	0	0	Upregulated	5.66E-03	3.26E-02	non-signifincat	non-signifincat	non-signifincat
isotig00672	1980	protein takeout-like	3.4E-55	---NA---	---NA---	cccp_droya ame: full=	4.4E-23	0	0	0	12	Downregulated	5.66E-03	3.50E-02	non-signifincat	non-signifincat	non-signifincat
isotig05057	380	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	12	0	0	0	Upregulated	5.66E-03	4.56E-02	non-signifincat	non-signifincat	non-signifincat
isotig05461	315	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	12	0	0	Upregulated	5.66E-03	4.11E-02	non-signifincat	non-signifincat	non-signifincat
isotig04921	389	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	14	4	0	1	Upregulated	6.08E-03	1.77E-02	non-signifincat	non-signifincat	non-signifincat
isotig04525	441	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	9	8	1	0	Upregulated	7.94E-03	1.27E-02	non-signifincat	non-signifincat	non-signifincat
isotig02922	706	isoform a	2.7E-26	---NA---	---NA---	pg12a_mouse ame: fu	1.8E-14	13	4	1	0	Upregulated	7.94E-03	2.29E-02	non-signifincat	non-signifincat	non-signifincat
isotig03603	572	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	8	2	Downregulated	8.07E-03	2.80E-02	non-signifincat	non-signifincat	non-signifincat
isotig04892	400	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	11	0	Downregulated	8.07E-03	4.74E-02	non-signifincat	non-signifincat	non-signifincat
isotig05441	320	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	11	0	Downregulated	8.07E-03	4.74E-02	non-signifincat	non-signifincat	non-signifincat
isotig05165	364	PREDICTED: hypothetical protein L(	5.7E-38	PREDICTED: Apis me	1.9E-12	---NA---	---NA---	0	11	0	0	Upregulated	8.07E-03	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05171	365	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	11	0	0	Upregulated	8.07E-03	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05628	248	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	11	0	0	Upregulated	8.07E-03	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig02225	964	glucose dehydrogenase	5.5E-67	---NA---	---NA---	dhgl_drops ame: full=	2.4E-56	0	1	9	6	Downregulated	1.04E-02	1.82E-02	non-signifincat	non-signifincat	non-signifincat
isotig04855	405	gag protein	1.4E-12	---NA---	---NA---	gagj_drome ame: full=	9.2E-13	19	1	3	0	Upregulated	1.12E-02	3.13E-02	non-signifincat	non-signifincat	non-signifincat
isotig01999	1119	ribosome-binding protein 1	3.2E-46	tribolium castaneum 1	7.5E-11	rrbp1_mouse ame: ful	7.7E-11	4	19	2	2	Upregulated	1.13E-02	1.71E-02	non-signifincat	non-signifincat	non-signifincat
isotig03491	585	heterotrimeric g protein alpha subuni	8.2E-96	anopheles gambiae p	2.9E-120	gnao_locmi ame: full=	3.8E-96	0	0	8	2	Downregulated	1.17E-02	4.07E-02	non-signifincat	non-signifincat	non-signifincat
isotig02960	697	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	0	9	Downregulated	1.17E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig02759	753	hypothetical protein TSTA_040370 [	1.1E-31	scedosporium apiosp	0.0E+00	---NA---	---NA---	0	0	0	10	Downregulated	1.17E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig02086	1043	retrotransposon protein	2.0E-45	eucalyptus grandis cc	0.0E+00	---NA---	---NA---	0	0	0	10	Downregulated	1.17E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig02848	724	mitochondrial ribosomal	9.1E-52	---NA---	---NA---	rm48_human ame: ful	7.9E-16	0	1	7	7	Downregulated	1.39E-02	3.21E-02	non-signifincat	non-signifincat	non-signifincat
isotig02160	997	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	1	0	5	8	Downregulated	1.39E-02	3.40E-02	non-signifincat	non-signifincat	non-signifincat
isotig02119	1023	contactin	1.5E-132	apis mellifera contacti	4.0E-77	cont_drome ame: full=	4.6E-102	0	1	10	4	Downregulated	1.39E-02	2.83E-02	non-signifincat	non-signifincat	non-signifincat
isotig03478	591	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	1	12	1	0	Upregulated	1.39E-02	4.93E-02	non-signifincat	non-signifincat	non-signifincat
isotig01744	1461	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	11	10	3	1	Upregulated	1.68E-02	1.87E-02	non-signifincat	non-signifincat	non-signifincat
isotig03545	581	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	4	17	2	2	Upregulated	1.68E-02	2.73E-02	non-signifincat	non-signifincat	non-signifincat
isotig02726	763	microtubule-associated protein 2	6.1E-46	nasonia vitripennis mi	1.0E-57	map4_rat ame: full=m	9.3E-14	8	39	4	7	Upregulated	1.68E-02	8.42E-03	non-signifincat	non-signifincat	non-signifincat
isotig03372	609	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	5	4	Downregulated	1.72E-02	4.57E-02	non-signifincat	non-signifincat	non-signifincat
isotig03383	603	nol1 nop2 sun domain family	2.5E-19	---NA---	---NA---	---NA---	---NA---	0	0	5	4	Downregulated	1.72E-02	4.57E-02	non-signifincat	non-signifincat	non-signifincat
isotig03804	539	mitochondrial ribosomal protein s33	2.2E-41	anopheles gambiae p	7.6E-26	rt33_mouse ame: full=	1.7E-24	0	0	5	4	Downregulated	1.72E-02	4.57E-02	non-signifincat	non-signifincat	non-signifincat
isotig04762	416	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	2	6	Downregulated	1.72E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04424	455	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	2	7	Downregulated	1.72E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05331	338	egl nine homolog 1-like	1.7E-63	nasonia vitripennis eg	2.7E-54	egln3_human ame: fu	1.2E-55	0	0	2	7	Downregulated	1.72E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig02775	748	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	2	8	Downregulated	1.72E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04517	443	charged multivesicular body protein	1.1E-20	culex quinquefasciatu	4.8E-21	chmp5_drome ame: fu	5.7E-14	0	0	8	1	Downregulated	1.72E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04985	389	e3 ubiquitin-protein ligase rnf25	4.7E-45	---NA---	---NA---	rnf25_bovin ame: full=	1.6E-22	0	0	8	1	Downregulated	1.72E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04534	440	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	1	8	Downregulated	1.72E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig01107	453	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	1	8	Downregulated	1.72E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05554	286	cdp diglyceride synthetase	6.8E-27	drosophila ananassae	5.3E-37	cdsa_drome ame: full	6.7E-28	0	0	1	8	Downregulated	1.72E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04952	391	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	8	0	Downregulated	1.72E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04994	386	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	0	8	Downregulated	1.72E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04933	395	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	0	8	Downregulated	1.72E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05496	307	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	0	8	Downregulated	1.72E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05639	233	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	0	8	Downregulated	1.72E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05648	219	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	0	8	Downregulated	1.72E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig01723	1516	programmed cell death protein 7	9.7E-43	---NA---	---NA---	pdcd7_human ame: fu	7.2E-24	1	0	11	6	Downregulated	1.78E-02	1.03E-02	non-signifincat	non-signifincat	non-signifincat
isotig05736	131	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	17	0	0	2	Upregulated	1.78E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig00883	563	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	1	9	3	Downregulated	1.86E-02	4.23E-02	non-signifincat	non-signifincat	non-signifincat
isotig00093	434	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	10	4	0	1	Upregulated	1.86E-02	4.95E-02	non-signifincat	non-signifincat	non-signifincat
isotig03514	581	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	4	9	0	1	Upregulated	1.86E-02	4.95E-02	non-signifincat	non-signifincat	non-signifincat
isotig04294	470	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	3	10	0	1	Upregulated	1.86E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig03509	584	mitochondrial ubiquitin ligase activat	1.9E-45	---NA---	---NA---	mul1a_danre ame: ful	2.3E-18	1	11	1	1	Upregulated	1.86E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04963	391	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	14	0	0	1	Upregulated	1.86E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig01822	1333	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	17	15	3	2	Upregulated	1.90E-02	3.88E-03	non-signifincat	0.814504763	0.774129535
isotig00164	1304	hypothetical conserved protein	1.8E-31	gryllus bimaculatus gl	2.1E-12	---NA---	---NA---	3	2	13	13	Downregulated	2.06E-02	1.13E-02	non-signifincat	non-signifincat	non-signifincat
isotig02740	755	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	17	14	2	3	Upregulated	2.19E-02	5.12E-03	non-signifincat	0.803310794	0.87143672
isotig02919	704	probable alpha-ketoglutarate-depenc	7.9E-55	xenopus laevis alkyla	9.5E-39	alkb5_xenla ame: full	1.4E-53	8	9	2	1	Upregulated	2.28E-02	2.60E-02	non-signifincat	non-signifincat	non-signifincat
isotig02425	870	Apolipophorin [Acromyrmex echinati	3.2E-50	---NA---	---NA---	aplp_locmi ame: full=	1.2E-12	4	12	1	2	Upregulated	2.28E-02	4.02E-02	non-signifincat	non-signifincat	non-signifincat
isotig02197	973	rna polymerase	2.4E-34	---NA---	---NA---	l_uuks ame: full=rna-	6.7E-24	0	1	7	5	Downregulated	2.52E-02	4.83E-02	non-signifincat	non-signifincat	non-signifincat
isotig02420	873	conserved hypothetical protein [Ajell	2.7E-13	---NA---	---NA---	unc89_caeel ame: full	3.3E-11	0	1	7	5	Downregulated	2.52E-02	4.83E-02	non-signifincat	non-signifincat	non-signifincat
isotig05438	321	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	8	5	1	0	Upregulated	2.52E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig02564	821	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	6	6	0	1	Upregulated	2.52E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig00755	1387	lipophorin receptor	0.0E+00	blattella germanica m	0.0E+00	ldlr_rat ame: full=low-	8.9E-100	1	11	0	1	Upregulated	2.52E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig01743	1471	rna-dependent rna polymerase	3.7E-44	---NA---	---NA---	l_rysv ame: full=rna-d	2.4E-37	0	2	7	12	Downregulated	2.54E-02	3.24E-02	non-signifincat	non-signifincat	non-signifincat
isotig02672	775	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	18	2	2	2	Upregulated	2.54E-02	4.14E-02	non-signifincat	non-signifincat	non-signifincat
isotig04255	473	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	18	2	1	3	Upregulated	2.54E-02	4.35E-02	non-signifincat	non-signifincat	non-signifincat
isotig02088	1040	aldose reductase	1.2E-152	tribolium castaneum a	2.9E-47	aldr_rat ame: full=ald	1.2E-121	5	9	21	37	Downregulated	2.55E-02	3.95E-03	non-signifincat	non-signifincat	non-signifincat
isotig04830	408	cornetto cg32386-pa	5.8E-13	---NA---	---NA---	---NA---	---NA---	0	0	4	4	Downregulated	2.59E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig03167	652	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	4	4	Downregulated	2.59E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig02867	721	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	5	3	Downregulated	2.59E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig03938	519	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	0	5	3	Downregulated	2.59E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04431	451	aldehyde dehydrogenase mitochond	4.3E-44	nomascus leucogeny	1.1E-54	al1a1_chick ame: full=	5.6E-41	2	31	2	5	Upregulated	2.63E-02	3.94E-02	non-signifincat	non-signifincat	non-signifincat
isotig00685	1668	small heat shock protein	2.2E-67	pteromalus puparum	1.3E-117	---NA---	---NA---	1	0	6	9	Downregulated	2.93E-02	1.82E-02	non-signifincat	non-signifincat	non-signifincat
isotig03294	624	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	9	7	2	1	Upregulated	2.93E-02	4.45E-02	non-signifincat	non-signifincat	non-signifincat
isotig05267	352	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	1	0	11	4	Downregulated	2.93E-02	2.09E-02	non-signifincat	non-signifincat	non-signifincat
isotig05570	281	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	5	10	1	2	Upregulated	2.93E-02	4.84E-02	non-signifincat	non-signifincat	non-signifincat
isotig04043	502	metalloprotease m41 ftsh	1.5E-94	culex quinquefasciatu	4.5E-79	yme1_caeel ame: full	1.1E-72	14	1	2	1	Upregulated	2.93E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05313	343	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	1	14	2	0	Upregulated	2.93E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04683	423	serine threonine protein phosphatase	9.9E-67	mus musculus protein	9.9E-93	2a5g_human ame: ful	3.3E-65	0	15	2	0	Upregulated	2.93E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig00351_isotig003	1582	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	15	41	5	10	Upregulated	2.98E-02	5.87E-03	non-signifincat	non-signifincat	non-signifincat
isotig01725	1512	major royal jelly protein 4	8.8E-49	---NA---	---NA---	yell_drosu ame: full=p	1.1E-23	1	10	17	29	Downregulated	3.18E-02	1.22E-02	non-signifincat	non-signifincat	non-signifincat
isotig02709	765	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	5	23	7	86	Downregulated	3.45E-02	3.84E-02	non-signifincat	non-signifincat	non-signifincat
isotig02096	1022	PREDICTED: similar to F59H6.5, pa	1.6E-60	arabidopsis thaliana c	1.7E-18	pif1_human ame: full=	3.2E-12	1	0	5	6	Downregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig02507	842	zinc finger protein on ecdysone puffs	2.3E-45	gryllus bimaculatus gl	1.3E-12	---NA---	---NA---	0	1	6	5	Downregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig00833_isotig008	1007	---NA---	---NA---	af297109helicoverpa	2.0E-17	---NA---	---NA---	0	1	4	7	Downregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig02845	726	dual specificity protein phosphatase	4.5E-27	anolis carolinensis du	1.3E-43	dus10_bovin ame: full	7.6E-22	5	6	1	0	Upregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04577	431	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	1	2	9	Downregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04270	473	sortilin-related receptor	4.7E-15	---NA---	---NA---	---NA---	---NA---	3	9	1	0	Upregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05083	376	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	3	9	1	1	Upregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04728	418	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	10	1	0	1	Upregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05229	357	PREDICTED: hypothetical protein L(	1.0E-12	---NA---	---NA---	---NA---	---NA---	1	10	1	0	Upregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05597	266	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	11	1	1	Upregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig02572	815	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	1	10	0	1	Upregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig03633	567	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	11	0	1	0	Upregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05511	304	plasma membrane calcium-transport	5.4E-48	sus scrofa ca++ plasn	1.0E-33	at2b3_human ame: fu	5.6E-45	11	0	1	0	Upregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig03520	578	isoform b	3.3E-29	PREDICTED: Apis me	1.7E-15	---NA---	---NA---	11	0	0	1	Upregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04894	401	isocitrate dehydrogenase	1.8E-59	drosophila erecta gg1	2.9E-99	idhc_sheep ame: full=	1.9E-53	0	11	1	0	Upregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05269	352	integrase core domain protein	8.8E-26	---NA---	---NA---	copia_drome ame: ful	1.7E-11	0	11	0	1	Upregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05527	298	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	11	0	1	Upregulated	3.45E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig02061	1057	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	4	10	0	2	Upregulated	3.79E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig02224	964	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	4	10	0	2	Upregulated	3.79E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig03980	509	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	2	2	11	Downregulated	3.79E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig01928	1189	dna helicase partial	1.2E-30	---NA---	---NA---	---NA---	---NA---	2	0	10	7	Downregulated	3.92E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04339	464	myosin heavy chain	9.7E-13	---NA---	---NA---	actnb_dicdi ame: full=	1.0E-12	13	5	1	3	Upregulated	3.92E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig03094	665	10 kda heat shock mitochondrial-like	2.8E-36	single read from an e	2.2E-27	ch10_schja ame: full=	3.9E-26	3	2	14	9	Downregulated	3.98E-02	2.62E-02	non-signifincat	non-signifincat	non-signifincat
isotig01817	1346	pyridine nucleotide-disulfide oxidore	3.8E-110	xenopus tropicalis pyr	2.9E-23	pyrd1_rat ame: full=p	5.2E-107	3	2	13	9	Downregulated	3.98E-02	3.09E-02	non-signifincat	non-signifincat	non-signifincat
isotig01435	3811	s-adenosylmethionine decarboxylase	4.4E-144	pediculus humanus c	7.4E-49	dcam_drome ame: ful	1.1E-104	121	140	16	72	Upregulated	4.20E-02	1.01E-02	non-signifincat	non-signifincat	non-signifincat
isotig01508	2265	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	10	13	27	Downregulated	4.28E-02	3.05E-02	non-signifincat	non-signifincat	non-signifincat
isotig04263	473	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	10	18	6	2	Upregulated	4.55E-02	3.01E-02	non-signifincat	non-signifincat	non-signifincat
isotig03521	582	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	2	20	1	4	Upregulated	4.73E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig00739	1138	transcription elongation factor spt6	1.7E-48	apis mellifera transcri	7.1E-62	spt6h_danre ame: full	4.8E-27	0	1	4	6	Downregulated	4.79E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig00845_isotig008	789	glutathione s-transferase d1	1.9E-59	locusta migratoria glu	4.3E-50	gstt1_drome ame: full	3.2E-59	1	0	2	8	Downregulated	4.79E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig03503	583	receptor-type tyrosine-protein phosp	1.3E-85	cotesia congregata le	2.9E-25	---NA---	---NA---	3	8	1	1	Upregulated	4.79E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig01380_isotig013	628	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	1	0	9	1	Downregulated	4.79E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05004	387	PREDICTED: hypothetical protein L(	2.1E-11	---NA---	---NA---	---NA---	---NA---	1	9	1	1	Upregulated	4.79E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05026	385	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	1	9	1	0	Upregulated	4.79E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig02547	825	thymocyte selection-associated high	8.0E-24	bombyx mori clone: fu	7.6E-22	tox4_ponab ame: full=	3.1E-12	1	9	0	1	Upregulated	4.79E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig03053	677	isoform b	9.0E-96	drosophila melanogas	9.7E-83	spg7_human ame: ful	5.0E-48	0	10	0	1	Upregulated	4.79E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05567	285	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	0	10	0	1	Upregulated	4.79E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04657	428	nadh:ubiquinone dehydrogenase	1.4E-33	---NA---	---NA---	---NA---	---NA---	13	7	3	1	Upregulated	4.82E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig03188	646	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	11	8	0	4	Upregulated	4.82E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig03546	582	cornichon protein	7.6E-70	strongylocentrotus pu	5.9E-72	cni_drome ame: full=	7.8E-63	1	2	0	18	Downregulated	4.82E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig01983	1129	ribonucleic acid binding protein s1	6.5E-29	mus musculus ribonu	1.3E-20	rnp1b_xenla ame: full	8.5E-29	0	2	5	11	Downregulated	4.91E-02	3.78E-02	non-signifincat	non-signifincat	non-signifincat
isotig02223	966	24-dehydrocholesterol reductase	1.1E-98	aedes aegypti 24-deh	1.4E-44	dhc24_human ame: fu	2.7E-88	11	5	3	1	Upregulated	4.91E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig03409	602	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	8	6	0	3	Upregulated	4.94E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig02623	796	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	5	8	2	1	Upregulated	4.94E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04649	427	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	5	8	0	3	Upregulated	4.94E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig04104	496	---NA---	---NA---	monodelphis domesti	8.5E-44	---NA---	---NA---	3	10	2	0	Upregulated	4.94E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig05154	363	serine threonine-protein kinase tao1	2.0E-28	culex quinquefasciatu	3.6E-53	---NA---	---NA---	14	0	0	2	Upregulated	4.94E-02	non-signifincat	non-signifincat	non-signifincat	non-signifincat
isotig00795	1274	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	6	2	17	17	Downregulated	4.96E-02	1.83E-02	non-signifincat	non-signifincat	non-signifincat
isotig01605	1795	chitin deacetylase 1	0.0E+00	drosophila yakuba ge	0.0E+00	---NA---	---NA---	4	5	21	12	Downregulated	4.96E-02	1.83E-02	non-signifincat	non-signifincat	non-signifincat
isotig01502	2310	pyruvate dehydrogenase	1.5E-179	tribolium castaneum p	2.5E-128	odpa_caeel ame: full=	5.5E-143	5	2	14	15	Downregulated	non-significat	3.00E-02	non-signifincat	non-signifincat	non-signifincat
isotig02610	800	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	11	13	1	5	Upregulated	non-significat	4.73E-02	non-signifincat	non-signifincat	non-signifincat
isotig01800	1370	sortilin-related receptor	2.2E-32	gryllus bimaculatus gl	1.5E-33	mysa_drome ame: ful	1.2E-12	9	21	6	2	Upregulated	non-significat	3.68E-02	non-signifincat	non-signifincat	non-signifincat
isotig03880	527	sensory appendage protein 1	9.5E-12	culex quinquefasciatu	2.4E-19	peb3_drome ame: full	1.1E-11	5	6	12	25	Downregulated	non-significat	2.49E-02	non-signifincat	non-signifincat	non-signifincat
isotig01453	2953	circadian clock-controlled isoform 1	4.2E-14	---NA---	---NA---	---NA---	---NA---	40	9	8	9	Upregulated	non-significat	4.27E-02	non-signifincat	non-signifincat	non-signifincat
isotig01786	1392	mitochondrial phosphate carrier prot	1.3E-169	gryllus bimaculatus gl	1.8E-165	mpcp_rat ame: full=pl	1.8E-155	5	10	22	27	Downregulated	non-significat	1.86E-02	non-signifincat	non-signifincat	non-signifincat
isotig01755	1453	s-adenosylhomocysteine hydrolase	0.0E+00	tetraodon nigroviridis	0.0E+00	sahha_xenla ame: full	0.0E+00	27	33	15	10	Upregulated	non-significat	3.89E-02	non-signifincat	non-signifincat	non-signifincat
isotig01511	2257	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	34	26	11	15	Upregulated	non-significat	4.33E-02	non-signifincat	non-signifincat	non-signifincat
isotig01544	2015	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	23	5	37	35	Downregulated	non-significat	3.66E-02	non-signifincat	non-signifincat	non-signifincat
isotig01312_isotig013	730	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	15	16	57	30	Downregulated	non-significat	1.58E-02	non-signifincat	non-signifincat	non-signifincat
isotig01484	2518	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	84	264	76	83	Upregulated	non-significat	4.63E-02	3.66E-02	non-signifincat	non-signifincat
isotig01310__isotig01	793	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	65	195	261	340	Downregulated	non-significat	3.14E-02	non-signifincat	non-signifincat	non-signifincat
isotig02308	919	---NA---	---NA---	---NA---	---NA---	---NA---	---NA---	193	641	1001	1010	Downregulated	non-significat	2.54E-02	non-signifincat	non-signifincat	non-signifincat