

Interpreting physiological responses to environmental change through gene expression profiling

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Summary

Identification of differentially expressed genes in response to environmental change offers insights into the roles of the transcriptome in the regulation of physiological responses. A variety of methods are now available to implement large-scale gene expression screens, and each method has specific advantages and disadvantages. Construction of custom cDNA microarrays remains the most popular route to implement expression screens in the non-model organisms favored by comparative physiologists, and we highlight some factors that should be considered when embarking along this path. Using a carp cDNA microarray, we have undertaken a broad, system-wide gene expression screen to investigate the physiological mechanisms underlying cold and hypoxia acclimation. This dataset provides a starting point from

which to explore a range of specific mechanistic hypotheses at all levels of organization, from individual biochemical pathways to the level of the whole organism. We demonstrate the utility of two data analysis methods, Gene Ontology profiling and rank-based statistical methods, to summarize the probable physiological function of acclimation-induced gene expression changes, and to prioritize specific genes as candidates for further study.

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Introduction

The phenotype of an organism is determined by the combined activities of thousands of genes that are coordinated both temporally and spatially. A goal of so-called 'omic' approaches is to understand complex biological systems by modeling the relationship between multiple measured attributes of biomolecular organization and the phenotype of the organism. Development of high-throughput methods means that large amounts of information can now be gathered at distinct levels of biological organization, allowing genotype, mRNA expression, protein, metabolic and physiological data, to be gathered for a particular organism under a specified set of conditions. Collecting these data is becoming increasingly simple and a rich resource of molecular information is available for the common laboratory model organisms (Bieri et al., 2007; Crosby et al., 2007; Nash et al., 2007).

Since phenotype results ultimately from the expression of genes and gene complexes, understanding patterns of gene expression evoked during changes in physiological state, or in response to environmental change, yields insights regarding the molecular basis of phenotype from the cellular to the whole

organism level. A key tool deployed in this research is the measurement of mRNA transcript levels by microarray hybridization (Gracey and Cossins, 2003). The microarray-based approach monitors the expression of many thousands of genes simultaneously, providing a broad view of the transcriptional changes that accompany alterations in physiological state.

The role of the transcriptome in physiological regulation

A significant challenge is how to decipher the large amounts of 'omic' data and then relate them to the phenotype of the study organism. To meet this challenge, a new approach to understanding complex biological systems, termed 'systems biology', has been proposed (Ideker et al., 2001a). A precise definition of systems biology is difficult, but generally speaking systems-based approaches aim to measure several sources of molecular information during genetic or environmental perturbations of a biological system, integrate these data, and then build predictions as to how the system might respond to a different perturbation. Thus, the better the

understanding of, and ability to model, a biological system, the better the predictions will agree with the experimental observations.

Systems-based interpretations of biological processes are revealing new insights into the role of the transcriptome in the regulation of phenotype. A common theme in most systems-based investigations is an effort to link gene or protein expression data with protein–protein and protein–DNA interaction data (Ideker et al., 2001b). The rationale behind this approach is that genes do not function in isolation and instead are components of wider networks of interacting molecules. As components of a wider system, the consequences of gene expression should be interpreted within the context of the behavior of the other molecules that participate in the biology of the organism. For example, systems-based analysis of gene expression has begun to explain why genes that are strongly differentially expressed in yeast stress experiments often turn out to have no discernable effect on stress sensitivity in deletion mutants (Birrell et al., 2002; Giaever et al., 2002). Using the yeast response to arsenic as a model, the analysis of deletion knockouts revealed that the genes that conferred the most sensitivity to arsenic were in pathways upstream of the arsenic detoxification pathways, while expression profiling identified genes that were members of downstream pathways that ultimately protect against toxicity but which share redundant functions, explaining why they have no phenotypic effect with deletion (Haugen et al., 2004). A similar conclusion was reached upon a system-based analysis of the DNA-damage response of yeast (Workman et al., 2006). So interpretation of gene expression data within the context of regulatory and metabolic networks suggests that gene expression profiling tends to interrogate the downstream effectors of biological responses.

A frequently asked question is whether changes in mRNA levels are a useful proxy for inferring changes in protein abundance? The scientific literature is replete with studies that have tried to address this question, most often by applying a combination of microarray and proteomic techniques to explore the concordance between mRNA and protein levels (Tian et al., 2004). A recent study provides the most definitive exploration of this relationship, and supersedes others by using an extremely accurate method to directly measure protein abundance in living cells (Newman et al., 2006). Using a collection of yeast strains in which each gene is expressed as a GFP-tagged fusion protein, GFP fluorescence was measured to profile the expression of each protein under different environmental perturbations and correlated with changes in the corresponding mRNA abundance. Use of fluorescence provided unprecedented resolution of protein abundance and revealed that mRNA abundance in 87% of genes changed >twofold, showing correlated changes in protein abundance. In a minority of cases, changes in protein abundance were observed in the absence of a change in mRNA level. The conclusion that can be drawn from these results is that mRNA expression profiling is an effective method to identify genes whose protein expression is regulated at the transcriptional

level, with the obvious caveat that proteomic techniques are required to identify post-translationally regulated genes. Indeed, a recent estimate assigns 73% of protein variance in yeast to transcriptional regulation (Lu et al., 2007), and so gene expression screens will not provide insights into the regulation of at least 25% of the proteome.

Constructing microarrays for non-model organisms

Array-based technologies are still the main platforms for undertaking large-scale gene expression screens. Arrays can be produced for any organism for which DNA sequence or nucleic acid material is available, and so in theory can be applied to any given organism. In my laboratory this has included constructing custom arrays for a variety of species including common carp (Gracey et al., 2004), the goby *Gillichthys mirabilis* (Gracey et al., 2001), golden-mantled ground squirrel (Williams et al., 2005) and, recently, for the colonial ascidian *Botryllus schlosseri* and the California ribbed mussel *Mytilus californianus*. Probably the greatest challenge to producing these species-specific ‘boutique’ or ‘bespoke’ arrays is generating the DNA probes that will be physically deposited on the microarray. Two major sources of probes are either PCR products or oligonucleotides. PCR products are generated either by targeted amplification of specific genes based on their DNA sequence, or alternatively by amplification of cDNAs that have been cloned into plasmids. As an alternative to PCR products, long oligonucleotides can be spotted on the array but their application is restricted to genes for which a DNA sequence is available.

Comprehensive sequence data are often limited for most non-model organisms, precluding the design of gene-specific oligonucleotides, and so cDNA microarrays produced in-house will remain the most viable option for most laboratories in the short-term. In this format, cDNA libraries provide a source of cDNAs, which are then amplified by PCR and the products spotted onto the array. Because the primers employed in the PCR are based on the vector sequences that flank the cloned cDNAs, this approach can be employed without prior knowledge of the sequence of the cloned cDNA. This means that sets of PCR-amplified cDNAs can be quickly and affordably created for almost any species.

The procedure for preparing PCR products from cloned cDNAs is simple and within the capacity of most molecular biology laboratories. Briefly, a cDNA library cloned in plasmids is transformed into *E. coli*, plated onto Luria-Bertani agar plates, yielding bacterial colonies that each represent a single cDNA clone. A small portion of each colony is then picked into either 96- or 384-well microtiter plates containing Luria-Bertani broth and propagated. Picking is done in a random fashion and thus the sequence and identity of the cDNA clone present in the host *E. coli* is unknown. The microtiter plates become the picked cDNA library with each coordinate in the plate representing the location of a discrete cDNA clone. Microtiter plates of clones can be copied and frozen, allowing the picked library to be stored indefinitely. Accurate tracking

of the plates of clones throughout the picking and archiving process is an essential step (Konno et al., 2001), since these plates will be the source for the next steps of PCR amplification, arraying and sequencing.

Ideally, we would like to be able to curate a set of cDNA clones that represents all the transcripts encoded by the genome of the organism we wish to study. An array fabricated with this clone-set, a so-called whole transcriptome microarray, would be invaluable since it would offer a global overview of how the expression of every gene in the organism is orchestrated under particular physiological or environmental conditions. However, creating comprehensive cDNA collections that cover the entire genome has proved a challenge. For most of the model organisms, laboratories around the world are collaborating in efforts to create complete cDNA collections, yet after years of work many cDNAs have remained elusive and the collections are still incomplete. The project to identify and sequence every transcript in the mouse genome is particularly noteworthy and an impressive range of strategies and tools has been deployed in this effort (Carninci et al., 2003; Okazaki et al., 2002; Carninci, 2007). With these problems in mind, it is important to consider the most effective strategy to create comprehensive cDNA collections and arrays for new species.

Normalized cDNA libraries

A number of strategies exist for the isolation and curation of cDNA clones for array fabrication. Since the expression pattern of the arrayed genes will be the guide for the interpretation of complex biological response, it is important that genes linked to the physiological process are well represented on the array. The first step to achieving this is to prepare the cDNA library using RNA isolated from the specific tissue(s) and physiological condition that will be the subject of the study. This greatly increases the likelihood that genes that are expressed under these conditions are present as cDNA clones in the library. For example, arrays developed to study the transcriptional response of gobies to hypoxia (Gracey et al., 2001), and killifish to thermal cycling (Podrabsky and Somero, 2004), were prepared from RNA isolated from animals exposed to these respective conditions. Still, the frequency with which the potentially interesting cDNAs are encountered in the library will depend on their abundance in the RNA sample, with highly transcribed genes being more likely to be picked from the library, whilst rare transcripts will be encountered with less frequency. For this reason every effort is made to ensure that rare genes are isolated during the picking of clones from the library. Two important procedures improve this situation, namely normalization and serial-subtraction. Normalization reduces redundancy within the library, bringing the frequency with which each gene is present in the library to within a narrow range, while subtraction enriches for genes specific to a particular environmental treatment, and serial subtraction increases the probability that new genes will be added to the clone-set (Carninci et al., 2000). In our experience, several rounds of serial subtraction is the most effective method of

creating cDNA libraries of low redundancy, and while these are time-consuming steps, the results justify the cost.

A universal goal of all of the major cDNA collection and annotation projects for model organisms is to isolate and sequence full-length cDNAs clones (Imanishi et al., 2004; Strausberg et al., 2002). A full-length cDNA clone is one in which the entire coding sequence is present together with the flanking 5' and 3' untranslated regions (UTRs). The contribution of full-length clones to understanding gene function cannot be understated. First, the complete cDNA sequence is useful for interpreting genomic sequences, where exons are interspersed with non-coding introns, and each gene may give rise to range of transcripts based on differential splicing. Thus, a full-length cDNA is evidence of the genomic sequence that was transcribed and of alternative splicing events (Zavolan et al., 2003). Second, identification of the translation initiation codon and the 5' UTR indicates the location of the promoter sequence of the gene, thus helping to direct exploration of the gene's regulatory elements. Third, and most importantly, knowing the sequence of the complete open reading frame of a gene greatly facilitates its functional annotation. In the first instance, it improves homology searches against the public databases and increases the likelihood that its putative function can be assigned based on homology. For cDNAs without recognizable homology to a known gene, characterization of the functional motifs of the protein may help predict its function (Okazaki et al., 2002). Furthermore, knowing the complete amino acid sequence of the encoded protein is important for comparative analyses that aim to understand differences in functional properties of orthologous proteins. For all these reasons, full-length cDNA clones are a valuable foundation upon which further investigations can be constructed.

One caveat with respect to using full-length clones as microarray probes is that their sequence may contain regions that share homology with other genes or contain repetitive sequence. These elements may lead to cross-hybridization between the cDNA and transcripts other than the target transcript. The degree to which these non-specific hybridization signals compromise array analysis is still unclear, but it appears that increasing the stringency of both hybridization and wash conditions can alleviate these problems (Drobyshev et al., 2003; Korkola et al., 2003). On the other hand, the tolerance of long cDNAs to base-pair mismatches can be turned into an advantage, since it allows nucleic acids from related taxa to be hybridized heterologously to a single species array (Renn et al., 2004; von Schalburg et al., 2005).

Subtracted cDNA libraries

Subtracted cDNA libraries are an alternative source of cDNAs for preparing array probes. Subtractive hybridization approaches are used to compare two RNA samples and yield populations of cDNAs enriched for genes that are specifically expressed at higher levels in one sample more than the other (Sagerstrom et al., 1997). Their main advantage is that they

purposefully enrich for cDNAs that are differentially expressed, so fewer cDNAs have to be screened to identify interesting genes. Screening fewer genes on an array improves statistical power since it reduces the number of type I errors that occur when multiple statistical comparisons are made. Subtraction also enriches for genes that are present at low abundance and may not have been discovered in the early stages of randomly picking clones from normalized libraries. In addition the cloned cDNAs tend to be biased towards the most unique gene-specific sequences making them ideal array probes in terms of their specificity. So arrays produced from subtracted libraries can offer some advantages but also present some unique problems with regard to analysis. Most microarray data normalization protocols are based on the assumption that the majority of genes on the array are not differentially expressed and that approximately equal numbers of genes are up- and downregulated (Quackenbush, 2001), but these assumptions may fail for arrays produced from subtracted libraries since spots may show a biased direction of differential expression (Oshlack et al., 2007). Therefore, subtracted libraries should be complemented with cDNAs from non-subtracted sources for microarray construction to alleviate this bias. Another problem is that most methods of cDNA subtraction generate cDNA fragments rather than intact complete cDNAs (Diatchenko et al., 1996), and identification of fragments by sequence homology is problematic for unsequenced organisms (Gracey et al., 2001), and converting fragments into full-length clones is time-consuming.

Oligonucleotide-based arrays and standardization

A problem that has plagued cross-laboratory and cross-platform comparisons of microarray datasets has been the comparison of the probe content of different arrays. Typically, the arrayed probes are annotated using gene names but the assignment of names to DNA sequence is imprecise and strongly dependent upon the sequence database that is used as the reference for the annotation. So as these databases grow and more sequence data come to light, the putative identity assigned to genes evolves and can be subject to revision. This ambiguity leads to real problems when studies have sought to extract matching probes sets across platforms, leading to greater than expected discordance between data derived from different platforms (Tan et al., 2003). Seeking to resolve this problem, recent work has revealed that gene expression data show much greater cross-platform and between-laboratory consistency if the array elements are treated in a sequence-orientated *versus* gene annotation-centered manner (Kuo et al., 2006). This suggests that standardization will only be achieved if probes are matched by DNA sequence instead of by gene name, and this will necessitate a switch to oligonucleotide-based probes for those organisms for which either complete or extensive DNA sequence is available. While arrayed cDNAs offer a cheap and accessible route to gene expression profiling, they suffer the problem that the arrayed cDNAs are often incompletely sequenced, and instead represented by a 5' or 3'

expressed sequence tag (EST), which prevents the adoption of sequence-orientated interpretation of the data. For this reason, array platforms developed for non-model organisms that support a large community of interested researchers are expected to gravitate towards the oligonucleotide array format to provide a degree of standardization across laboratories in the community.

Oligonucleotide probes provide additional advantages beyond that of standardization. Most importantly, oligonucleotide probes can be designed to distinguish between genes with high degrees of sequence similarity (Hughes et al., 2001). This is particularly important given the complexity of the transcriptome, which may include transcripts that are alternatively spliced (Zavolan et al., 2003), antisense (Kiyosawa et al., 2003), allele-specific (Yan et al., 2002) or non-coding (Okazaki et al., 2002). The ability to explore the function of these transcripts initially through an understanding of when and where they are expressed will be dependent on the discriminatory power offered by oligonucleotide arrays.

Cross-laboratory and cross-platform standardization is only relevant if gene expression datasets are shared and made available in public databases. Most journals, including *The Journal of Experimental Biology*, require that gene expression data are submitted to one of the two public databases, either ArrayExpress (Parkinson et al., 2007), or NCBI GEO (Barrett et al., 2007). In the past we have found that submitting data to both these repositories was cumbersome and required informatics support in order to organize massive amounts of data into the format required for compliance. Realising that the complexity of the submission process was an obstacle to submission and full disclosure of expression data, the public databases have recently introduced a spreadsheet-based submission format (Rayner et al., 2006). This simple tabular format is similar to the one used by most gene expression analysis software packages, meaning that submission of expression data should be within the capability of any research group with the ability to generate and analyse microarray expression data. Removal of this impediment should streamline the submission and publication of expression data generated for non-model organisms, opening up the field to laboratories with limited informatics capacity. Accordingly, it is hoped that submitting new expression data to public databases will become as routine a step in microarray investigations as preparing high quality RNA.

High-throughput sequencing

A number of approaches have been developed recently that interrogate the transcriptome through very high-throughput sequencing, simultaneously facilitating gene discovery as well as generating a comprehensive view of transcript abundance without prior knowledge of gene sequence. In principle, these approaches should have considerable utility for transcriptome-based investigations into non-model organisms. In 'massively-parallel signature sequencing' (MPSS), the most-established of these high-throughput methods (Brenner et al., 2000), hundreds

of thousands of short gene-specific signature sequences are generated from a huge array of cDNAs that are bound to beads, and the frequency with which each sequence is detected provides a measure of the abundance of each gene in the transcriptome (Hoth et al., 2003; Jongeneel et al., 2003). MPSS is analogous to the established sequencing-based technique of Serial Analysis of Gene Expression (SAGE) (Velculescu et al., 1995), but MPSS offers much greater sensitivity because it interrogates many more sequences per sample (typically $>1 \times 10^6$ sequences in MPSS versus $<1 \times 10^5$ in SAGE) in a fraction of the time. This depth of sequencing means that transcripts that are present at extremely low levels can be detected and quantified in theory, if sufficient numbers of sequences are collected. In model organisms, the short signature sequences can then be mapped onto the genomic sequence, but in unsequenced organisms the genes must still be identified on a gene-by-gene basis using PCR primers based on the signature sequences. Despite this shortcoming, MPSS was used to investigate gene expression patterns underlying growth heterosis in oysters whose genome has not been sequenced, revealing that ribosomal proteins are differentially expressed, and suggesting an important role for protein metabolism in heterosis (Hedgecock et al., 2007). Finally, access to most of these technologies is only available through commercial companies, meaning that it may prove financially restrictive to use them for gene expression profiling. Instead, it is anticipated that high-throughput sequencing approaches will fulfill a pivotal role in gene discovery, by providing a benchmark of transcript abundance in selected RNA samples and the identification candidate genes whose expression can then be profiled using more affordable methods.

Gene expression data interpretation

A significant challenge arising from large-scale gene expression profiling experiments is how to interpret the resulting data. Opinions on how a particular dataset should be examined differ widely and there is unlikely to be a single perfect approach to extract all the useful insights from a large dataset. Testament to this is that there are many more publications describing the analysis of expression datasets than there are papers presenting original expression data. So as data analysis methods evolve and previous protocols are superseded, favorite expression datasets are mined repeatedly for more biological insights.

In the course of our investigations into the transcriptional response of carp to environmental cold (Gracey et al., 2004) and hypoxia (Fraser et al., 2006), we have adopted two different computational approaches to extract novel insights into the physiological response of carp to these perturbations. Our choice of methods was driven by the data: cold acclimation caused a substantial proportion of the transcriptome to be differentially expressed and this complexity prompted us to adopt a holistic interpretation method with an emphasis on the detection of biological themes within the expression data. In contrast, the transcriptional response to hypoxia was far more

constrained in terms of the number of genes that were expressed, and so we adopted a simple statistical method to identify genes that exhibited large changes in expression as candidates for follow-up studies.

Metabolic reprogramming during cold-acclimation

To generate new insights into the molecular processes that underlie cold-acclimation, we subjected common carp to a stepwise cooling regime from 30°C down to 10°C over 3 days and differential gene expression patterns across seven tissues were determined by hybridization to a custom carp microarray (Gracey et al., 2004). Interpreting the functional role of the complex changes in gene expression that accompany cold-acclimation response is a challenging task, and requires computational methods that transcend from individual genes to interpretations based on biological processes. Recent trends in gene expression analysis have indicated that the interpretation of expression data can be greatly improved if new data, instead of being analyzed in isolation, are interpreted within the context of a larger collection of curated sets of genes. In this approach, sets of functionally related genes are compiled as already characterized 'genesets', allowing expression data to be represented in terms of the behavior of these known sets of genes. The most common method of grouping genes is according to the Gene Ontology terms with which they are annotated: Gene Ontology, or GO, is a structured vocabulary that seeks to assign a consistent annotation to genes according to the biological function they perform, the biological process in which they involved, and their cellular or extracellular location (Ashburner et al., 2000). A variety of computational methods are then applied to test whether there are more genes associated with a GO term or biological pathway within a list of differentially expressed genes than would be expected by chance, to yield a heuristic measure of enrichment.

This approach has the ability to detect coherent changes in the expression of sets of genes that may be hidden when considering the expression patterns of individual genes in isolation. For example, analysis of the expression signatures of muscle samples from diabetics identified that oxidative phosphorylation genes were coordinately down-regulated in diabetes, despite the fact that the decrease in their expression was modest, just 20% (Mootha et al., 2003). Since the same ontology is applied universally to genes from all organisms, it allows gene expression datasets from different species to be compared from a thematic rather than from an orthologous gene perspective. For example, shared metabolic programs associated with aging were identified in *Drosophila* and *C. elegans* by identifying over-represented GO terms in the lists of genes that were differentially expressed during the onset of aging in each organism (McCarroll et al., 2004).

For the analysis of the carp cold-acclimation dataset presented here, one (Subramanian et al., 2005) of the many methods (Dennis et al., 2003; Zeeberg et al., 2003; Zhong et al., 2004) for detecting biological themes in sets of differentially expressed genes was applied to the data to

Table 1. *Genes that contribute most significantly to the upregulation of overall 'protein folding' gene expression in cooled carp tissues*

GO:0006457 Protein folding	Function
FK506-binding protein 1A	cis-trans prolyl isomerases
FK506-binding protein 3	
FK506 binding protein 9 precursor	
GrpE protein homolog 1, mitochondrial precursor	Molecular chaperones and facilitators of protein folding and membrane translocation
GrpE protein homolog 2, mitochondrial precursor	
Prefoldin subunit 2	Binds nascent polypeptides and promotes folding
ATP-dependent Clp protease ATP-binding subunit ClpX-like	Protein chaperone
DnaJ homolog subfamily A member 3, mitochondrial precursor	Prevents protein aggregation
Heat shock protein HSP 90-beta	Molecular chaperone
T-complex protein 1, alpha subunit	
T-complex protein 1, beta subunit	Subunits of the chaperonin containing
T-complex protein 1, eta subunit	TCP1 complex that assists in the folding
T-complex protein 1, gamma subunit	of newly translated proteins in cytosol
T-complex protein 1, zeta subunit	

for the profound hypoxia tolerance exhibited by carp. It is noteworthy that myoglobin protein was detected in the liver of fish living under well-oxygenated conditions, suggesting that myoglobin has a functional role in liver that goes beyond that of its putative role in hypoxia-adaptation. The detection and then hypoxia-induction of myoglobin was a completely unexpected discovery and exemplifies the use of gene expression screens in combination with gene-prioritizing statistical approaches to uncover novel aspects of physiological responses to environmental change.

Applying rank-based statistical methods to prioritize genes for further study has proved useful in other investigations of physiology. For example, gene expression profiling of leptin-regulated genes in the liver of obese *ob/ob* mice (Cohen et al., 2002) identified stearoyl-CoA desaturase as the top ranked gene under regulation, leading to follow-up studies that demonstrated that the desaturase has a key role in fat deposition and that mutations in desaturase promote weight loss (Ntambi et al., 2002). Using a custom microarray prepared for Darwin's ground finches, calmodulin was identified as the regulatory gene whose expression was most correlated with peak length across species (Abzhanov et al., 2006). This discovery prompted further studies, first to validate that there was more calmodulin protein expressed in the beaks of finches with

elongated beak morphology, and then to demonstrate that manipulating calmodulin expression in the beaks of chickens could modify morphology. These two studies demonstrate the usefulness of using rank-based criteria for prioritizing specific genes identified in gene expression screens for further physiological or evolutionary investigations.

Conclusions

Microarray-based gene expression profiling is just one of a growing number of '-omic' approaches but it has already had a significant impact on investigations into the physiological responses to environmental change. A particular strength of the technique is that it is relatively simple to construct a microarray for any organism, and access to the equipment needed to make and screen arrays is now available at most university core facilities. Studies to date have indicated that properly designed and executed gene expression screens can reveal new insights into physiological responses and can highlight aspects of biological responses that have perhaps been overlooked. Increasingly, gene expression data are being complemented with metabolic, proteomic and molecular interaction data, which together present an unprecedented holistic view of the machinery of the cell across several layers of complexity. The

Table 2. *Ranked list of statistically significant genes induced by hypoxia in carp liver*

Gene rank	Day 5 hypoxia at 30°C	Day 5 hypoxia at 17°C
1	Myoglobin	Hemopexin precursor
2	Myoglobin	Differentially regulated trout protein 1
3	Unclassifiable EST	Vitelline membrane layer protein 1
4	Cofilin	Unclassifiable EST
5	Warm-temperature acclimation protein	FLJ00246 protein
6	Hemopexin precursor	GTP-binding protein hflX
7	Unclassifiable EST	Fatty acid binding protein A
8	TFIIH basal transcription factor	Myoglobin
9	Death effector domain-containing 1	Cofilin
10	Unclassifiable EST	Unclassifiable EST

challenge of integrating these diverse data sources will be met by new computational tools that seek to understand and model biological systems and ultimately to predict how they will respond to environmental and physiological perturbations.

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