The Stress-Response Network in Animals: Proposals to Develop a Predictive Mathematical Model

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Abstract: Increasing evidence indicates that numerous genetic pathways responding to environmental stress in animals are regulated co-ordinately as well as independently. These stress-response systems should therefore be viewed in holistic terms as a network. As such, their behaviour is susceptible to mathematical modelling using a systems biology approach. This review outlines relevant evidence and describes a newly launched project to develop just such a model using stress-response data from multiple transgenic strains of *C. elegans* and *D. melanogaster*. We hope that our eventual model will be capable of predicting the effects of simple stressor mixtures with reasonable accuracy. To maximise the effectiveness and scope of this model, we appeal for help from colleagues to share reagents and data relevant to this project. We also present preliminary data where RNA interference has implicated the key transcription factor DAF-16 in an unexpected up-regulation of *cyp*-34A9 reporter expression by high cadmium.

1. STRESS RESPONSES AND MIXTURE TOXICITY

In multicellular organisms, the defensive cellular responses evoked by environmental stresses do not result from simple linear pathways, but rather from a network of interlinked pathways with multiple outputs. This makes it difficult to predict the biological effects of multiple stressors acting together, even though this is the normal situation for industrial pollution of soil or water, where several different contaminants are usually present together. There are few studies and no useful predictive models describing the molecular responses of multicellular organisms to several toxicants acting in concert. This is essentially a systems biology problem, requiring integration of complex molecular and toxicological information. Under the auspices of a Major Award from the UK-India Education and Research Initiative (UK-IERI), we intend to develop an *in silico* model describing the principal elements of a consensus stress response network (SRN) and its *in vivo* responses to single stressors, using data from two invertebrate model systems, the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*. This model will be used to predict the likely SRN responses to stressor mixtures, and such predictions will then be tested experimentally in both species so that the model can be refined accordingly. Since the SRN core pathways are highly conserved among animal taxa, general features of this model should find wider application in ecotoxicology. The dynamic aspects of this model, and in particular its ability to integrate multiple toxicant inputs for predicting likely SRN outputs, distinguish this from a simple map of the regulatory gene-circuits comprising the SRN.

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2. THE *C. ELEGANS* STRESS-RESPONSE NETWORK

The free-living soil nematode, *C. elegans*, is particularly suitable for joint *in vivo* and *in silico* studies – thanks to its small size, anatomical simplicity, ease of culture, rapid life-cycle, unrivalled genetics, complete genome sequence and sophisticated post-genomic technologies (e.g. RNAi) [1]. *C. elegans* stress-response pathways are studied using molecular biomarkers such as heat-shock proteins [2-5] or metallothioneins [6], and in terms of behavioural or life-cycle parameters such as fertility, growth, motility or feeding [7-9]. *C. elegans* is used in standard ASTMS tests for soil and water pollution [10, 11], as well as for sediment testing [12].

Several *C. elegans* stress-response pathways are influenced by the *daf*-2 insulin-like signalling pathway that regulates lifespan [13]. DAF-2 signalling down-regulates the FOXO transcription factor DAF-16, whose targets include the small heat-shock genes (also regulated by HSF-1) [14], the *mtl*-1 metallothionein gene, the *daf*-9 and *cyp*-34A9 (*dod*-16) P450 genes, and the mitochondrial *sod*-3 superoxide dismutase gene (whose product helps to inactivate reactive oxygen species, ROS). Each of these DAF-16 targets contributes slightly towards the overall lifespan extension conferred by DAF-16 up-regulation [13]. Although the heat-shock factor HSF-1 targets inducible heat-shock genes at elevated temperatures, it also has other functions, as revealed

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by its wide-ranging RNAi phenotypes [14, 15]. These multi-pathway links are confirmed by the presence of DAF-16 binding sites [14] as well as ethanol- and hypoxia-response elements [16] within the *hsp*-16.1 promoter, besides the previously identified HSF-1-binding HSEs [17]. These regulatory links show how a single gene can respond to multiple stressors, and how a single stressor can influence multiple outputs (ROS metabolism, metal resistance, chaperone activity and lifespan). Other genes affecting multiple stress responses include the *cep*-1 p53 orthologue [18].

Several partially independent sub-networks (SNs) can be distinguished within the worm SRN, each regulated principally by one major transcription factor (TF); the heat-shock SN is activated by HSF-1, the oxidative-stress SN by zygotic SKN-1 [19], the metallothionein SN by ELT-2 plus metal-induced derepression [20], and the hypoxia SN by HIF-1 (heterodimerised with ARNT) [21]. The primary TF in each SN can be ablated by RNAi, which should facilitate independent modelling of the corresponding SNs. Gene-array studies have shown that DAF-16 primarily up-regulates broad-spectrum detoxification processes [22], including members of the P450, short-chain dehydrogenase/reductase (SDR), UDP glucuronosyltransferase (UGT) and glutathione S-transferase (GST) gene families. This general pattern of response is seen in *Drosophila* and mouse as well as in *C. elegans*, although lineage-specific diversification of each gene family has occurred within these different animal taxa, such that the genes up-regulated in one species are not necessarily orthologues of those up-regulated in another [23].

3. THE *D. MELANOGASTER* STRESS-RESPONSE NETWORK

Despite its undoubted convenience for ecotoxicological as well as developmental and genetic studies, *C. elegans* is an organism veritably bristling with defences! Most if not all of its stress-response pathways show evidence of duplication and diversification, perhaps as a consequence of its opportunistic lifestyle as a compost bacterivore. Routinely, it has to cope with osmotic and hypoxic stresses (e.g. when waterlogged), and its natural environment contains multiple metals, organic breakdown products from decomposition processes, and a range of bacterial toxins in its food. This complexity suggests that an additional (and preferably simpler) invertebrate model is also required for this model-building project, particularly if valid extrapolations are to be made for vertebrate systems. An ideal model organism for this purpose is the fruit fly, *Drosophila melanogaster*, which has a smaller set of stress-response output genes and thus a simpler SRN. Searching the Flybase and Wormbase databases reveals that flies have <20 P450 genes and ~10 *gst* genes compared to >80 and 45, respectively, in *C. elegans*. Amongst other genes in the oxidative stress pathway, *C. elegans* has 5 superoxide dismutases (3 Cu/Zn cytosolic and 2 Mn mitochondrial SODs) and 3 catalases compared to just 2 SODs (1 of each) and 1 catalase in *D. melanogaster*. These differences point to a simpler basal stress-response network in the fly as compared to the worm. Based on data acquired so far (in the laboratories of DdeP and DKC) using identical stressors, it is likely that *Drosophila* will prove more sensitive to most if not all toxicants – perhaps reflecting the sophisticated defence systems which have evolved in *C. elegans*. One advantage of studying two well-characterised invertebrate models in parallel is that both the similarities and differences in response to particular toxicants will be highlighted. The former may point to highly conserved aspects of the SRN (which may well also extend to vertebrates), whereas the latter may repay further investigation and comparison with published studies on vertebrate responses (to determine whether *Drosophila* or *C. elegans* provides a better model for that part of the SRN). It is worth reiterating that the approach proposed here – involving multiple SRN output genes, multiple doses/time-points and multiple toxicants (both singly and in combination) – is simply not feasible in any vertebrate system. Therefore it will be important to examine carefully how far one can extrapolate predictions of likely mixture toxicity from an invertebrate model to vertebrates (including humans).

4. CONSTRUCTION OF THE INITIAL MODEL

Current knowledge of the *C. elegans* SRN encompasses an extensive parts list and at least the outlines of a topology or wiring-diagram model [24]. We know the principal effector genes in each stress-response pathway, and in most cases understand their regulation by stress-activated TFs. However, subtler aspects of effector gene regulation by multiple stressors are only now emerging (e.g. for *hsp*-16.1) [16]. Our aim is to build an understanding of the underlying control logic [24], initially of each SN separately, and ultimately of the whole SRN. We have started by curating and collating extant knowledge, so as to generate a gene-circuit model that summarises the known SRN – a process successfully applied to mesendoderm specification and heart development [25, 26]. Each link in the network must satisfy 3 criteria. First, the appropriate TF(s) should have expression patterns consistent with the proposed interaction. Second, experimental perturbation of an upstream TF should evoke an appropriate response from downstream target genes. Third, evidence that the upstream TF binds directly to the target gene promoter is required from other studies of the regulation of that gene. Our initial network model will necessarily be incomplete and will be continuously refined during the project to reflect new data. This will allow construction of a mathematical model, initially describing SNs within the SRN, but working towards a complete model. Our *in silico* model will incorporate regulatory cross-links between pathways and SNs as they emerge from the experimental data, and will be constructed as a dynamic model describing changes in effector gene expression under various types of stress.

5. EXPERIMENTAL APPROACH

Gene-arrays provide one obvious way to explore these stress-responses [27-33]. Gene-array studies of stress-responses in budding [28] and fission [29] yeasts show that overlapping stressor-specific patterns of gene regulation are the norm – superimposed on a core stress response (CSR) involving multiple metabolic genes, which is common to most stressors. The CSR is regulated by MAP kinase signalling *via* ATF in fission yeast [29], but by several stress-specific pathways in budding yeast [28]. Gene-array studies can provide complete coverage of genome-wide changes in gene expression in response to specific stressors [e.g. 30-33], but are limited by expense, insensitivity to small changes, and sometimes poor reproducibility. Such considerations effectively preclude the routine testing of multiple stressors

at different doses and time-points – let alone investigating varied combinations of such stressors.

Instead, our experimental data will come mainly from stress-reporter expression assays. We are currently testing a series of single toxicants, but will later progress to simple mixtures of 2 or 3 such toxicants. This approach was originally pioneered using *hsp*-16 and *hsp*-70 reporters [3-5] as biomarkers for both aquatic [34] and soil [35] contamination. Our list of test toxicants will include several metals as well as a variety of widely used pesticides acting on different targets. Currently available stress-inducible reporter strains in *C. elegans* include: *hsp*-16.2 [36], *sod*-3, *cep*-1 [18], *cyp*-35A2 [37], *gst*-1 [38], *mtl*-1/ *mtl*-2 [6] and *hsp*-16.1 [39] strains. This range of reporters has been greatly expanded thanks to the Baillie group's pioneering genome-wide GFP reporter project [40] to include:- *hsp*-60, *gst*-4, *sod*-1/-2/-4, *ctl*-1/-2, *skn*-1, *hsf*-1, 4 P450 genes (*cyp*-29A2/-31A3/-34A9/-35A3) [37] and 3 glutathione peroxidase genes, plus genes involved in DNA repair. However, several large gene-families are involved in *C. elegans* stress responses (~45 *gst*, >80 *cyp* and >10 glutathione peroxidase genes). From these, we have chosen representatives that are strongly inducible by stress. *C. elegans* GFP reporters allow *in vivo* monitoring of expression in real time using a microplate fluorometer, whereas l*acZ* expression can only be assessed *post mortem*.

6. TRANSCRIPTION FACTOR NODES

Studies to elucidate gene-regulatory networks [e.g. 41-43] have emphasised the key role of transcription factor (TF) nodes affecting the expression of multiple downstream effector genes. One recent study modelled the *C. elegans* gene-regulatory network involved in vulval differentiation, using a combination of TF mutants and GFP-reporter constructs for a range of cell-type-specific effector genes [43]. It is important to know the level and activity of key TFs at these nodes, though it may not be the absolute level of a TF but rather its activity (influenced by ligand-binding, phosphorylation etc) or subcellular location (nuclear versus cytoplasmic) [44] that influences downstream gene expression. To probe the regulatory role of TF nodes in SRN pathways, we will ablate the function of selected TFs by feeding RNAi (e.g. for HSF-1) [45]. The efficacy of RNAi for each TF will be checked by monitoring reporter expression in TF-promoter::GFP fusion strains. Since our main focus is on transcriptional control of the SRN, we do not intend to measure the activity of upstream signalling pathways that activate or repress key TFs. In many cases (e.g. MAP kinase or EGF-like signalling), these pathways are well described and can be incorporated into our *in silico* model (e.g. the same insulin-like pathway inhibits both SKN-1 and DAF-16) [46] – but in other instances, signals upstream of known TFs remain obscure (e.g. endogenous ligands for steroid receptor-related TFs). Ablation or down-regulation of one SN may also affect the expression of other SNs *via* cross-talk or compensation between pathways. This should emerge from the RNAi studies, providing evidence for cross-links in the underlying genetic networks. For each of the worm SRN-output genes, we will also compare corresponding genomic sequences between *C. elegans* and *C. briggsae* (related nematodes that diverged ~100 mya) [47], focussing on the regulatory regions [48] driving our reporter genes and looking for known TF binding sites. Such cross-species bioinformatic comparisons can also reveal conserved sequence blocks likely to represent TF-binding sites [49]. Algorithms [e.g. 50] are available to identify additional regulatory elements for co-regulated genes.

7. MATHEMATICAL MODELLING OF THE SRN

The complexity of the SRN as a whole can be adequately addressed only by a systems-biology approach exploiting mathematical tools to generate *in silico* models that encompass the behaviour of individual network components (e.g. SNs) and their integration into a multi-scale formulation of the overall SRN suitable for hypothesis generation and testing, from which *in vivo* responses to multiple stressors should arise as emergent properties. If such models are to be genuinely predictive, significant quantitative information will be required with regard to network properties. Our experimental programme is carefully structured to enable such information to be extracted, and we hope that our model design will embody a level of complexity commensurate with the development of viable and tractable models that encompass the essential biology without becoming drowned under a flood of extraneous detail.

Even so, the complexity of SRN is such that reliable intuition about how different pathways interact is difficult to establish, and reductionist approaches based on treating each individual SN in isolation would fail to address the central systems-biology questions as to how the SRN functions as a whole [cf. 51, 52]. Our point of departure is the simplifying assumption that each SN (centred on one TF) functions largely autonomously within the overall SRN. Although this is an over-simplification, it has the methodological advantage of breaking down the overall SRN into more tractable SN units; novel cross-links between these SNs should emerge as the experimental data accumulate. As a first step, we are modelling two of the core SNs, namely the heat-shock response (centred on HSF-1) and the oxidative stress response (centred on SKN-1); other SNs will be added to the core model at a later stage. Reporter expression data for incorporation into the model will include both quantitative (*n*-fold induction relative to zero controls) and dynamic (rate of increase per unit time) information across a range of test doses. Equilibrium and kinetic parameter values associated with each SN can be obtained from the published literature, directly from our experimental data, or inferred indirectly during model exploration and validation. This is a pioneering case study of how state-of-the-art modelling procedures can underpin the development of truly predictive biology.

Insights from both SNs (above) will enable biologically well-grounded models to be developed, initially in the form of nonlinear ordinary differential equations describing the levels of output gene expression within SNs and key TF concentrations, and subsequently the inferred cross-talk between different SNs. By correlating the model predictions against experimental data, model refinement can be pursued, both qualitatively (e.g. introducing further interactions between pathways) and quantitatively (obtaining reliable estimates of the key parameters). Our model(s) will be investigated by:- (i) detailed computational simulations (including sensitivity studies on the role of various parameters), (ii) dynamical systems approaches to gain quantitative understanding of the mechanisms underlying observed behaviour and (iii) application of asymptotic treatments to provide simpler model for-

mulations which still capture the data in a quantitative way, allowing the dominant mechanisms to be uncovered in a systematic fashion.

Our initial SRN model, based on stress-responses to single toxicants, will enable us to predict the likely effects of simple 2-component mixtures. When testing such mixtures, we expect that our initial model will require substantial modification to accommodate the new experimental data, but we are optimistic that less and less adjustment should be required as the model develops. Thus the initial model will be developed and improved through an iterative process of model-based prediction, experimental testing, and subsequent refinement. Model development should not be constrained by the complexity of the mathematics (stochastic, delay and spatio-temporal model formulations will be brought into play, if needed), but only by the desire to provide a realistic description of the biology.

8. A CASE STUDY: *cyp*-34A9 AND *daf*-16

During the early stages of this project, we were somewhat surprised to observe the induction of a *cyp*-34A9::GFP reporter (strain BC20306) at high concentrations of several heavy metals (shown for cadmium in Fig. (**1**), but also seen for copper and for zinc). Cytochrome P450 genes are more typically induced by xenobiotics (33), but cannot detoxify or sequester metals. However, previous studies have identified several *cyp* genes as targets directly up-regulated by the DAF-16 transcription factor [13, 22, 23], and one such target is *cyp*-34A9 (*dod*-16) [13]. This gene is separated from its nearest 5' neighbour *cyp*-34A10 by less than 300 bp of intergenic sequence, and the integrated transgene construct in strain BC20306 contains only 251 bp of upstream *cyp*-34A9 promoter sequence (accessible as supplementary Fig. **2** at www.bentham.org/totoxij). Even so, this short region includes a DAF-16 consensus binding site (CATTGT, spanning positions -187 to -191 relative to the transcriptional start site) as well as likely binding sites for other transcription factors. Since RNAi against DAF-16 abolishes the induction of *cyp*-34A9 by cadmium (Fig. **1**) – as well as reducing expression of a *daf*-16::GFP transgenic strain (TJ356; data not shown) – we conclude that cadmium induction of *cyp*-34A9 expression is probably mediated through DAF-16 binding to the previously identified upstream site [13]. The *cyp*-34A9 promoter contains a direct repeat of the nGAAn motif, but our *cyp*-34A9::GFP reporter strain shows poor heat inducibility at 35ºC, suggesting that this is not a functional HSF binding site. By contrast, the upstream promoter of *cyp*-35A2 contains a closer match (AAGCTCTT) to the HSF consensus binding site at around -100; the fact that a *cyp*-35A2::GFP reporter strain [37] shows strong heat inducibility at 35ºC suggests that this site may well be functional, though RNAi against HSF [45] will be needed to confirm this suggestion.

9. COMMUNITY INVOLVEMENT IN THE SRN PROJECT

This project has ambitious aims, and we do not expect our 3-year study to generate a definitive model whose predictions are invariably accurate. Nevertheless, even a crude model whose predictions roughly approximate to reality for most mixtures tested would still be a significant advance on our current predicament – where mixture toxicity remains largely unpredictable. The more output genes and stress-response data that can be fed into our *in silico* model, the more accurate its predictions will be. For this reason, we wish to appeal to all stress biologists, ecotoxicologists and molecular biologists working on stress-inducible genes in *C. elegans* or *D. melanogaster*, to make contact with the authors and to share new findings (e.g. advance online publications) and relevant reagents. We are aware that the selection of transgenic stress-reporter strains available to us is limited in scope, and that other teams have developed similar strains with proven responsiveness to particular chemical

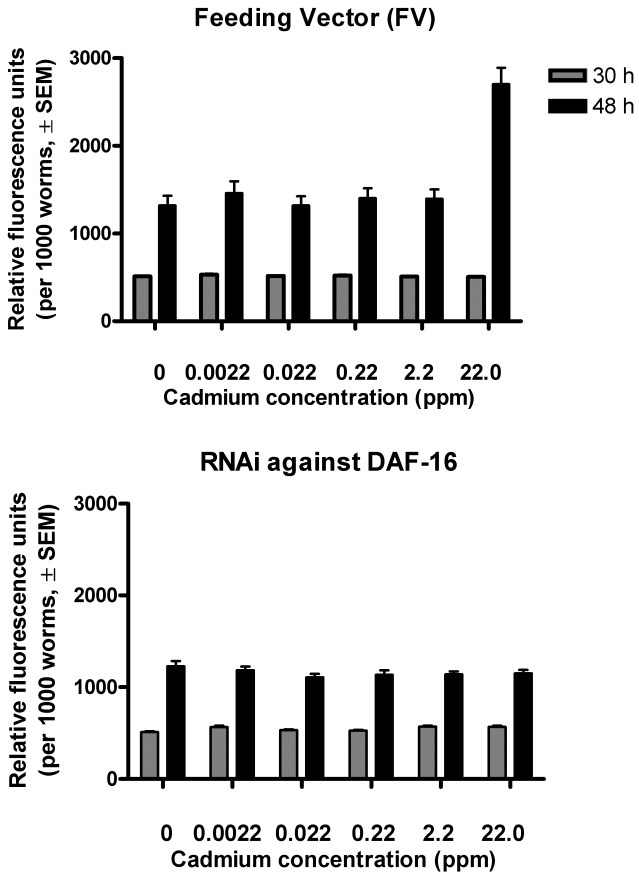

Fig. (1). Effect of RNA interference against DAF-16 on cadmium induction of cyp-34A9::GFP.

Aliquots of ~1000 young adult BC20306 (carrying 251 bp of upstream promoter from the c*yp*-34A9 gene [B0213.15] fused to a GFP coding sequence) [40] were dispensed into 24-well plates after feeding overnight on *E. coli* expressing IPTG-induced dsRNA from either a feeding-vector-only control strain (L4440) or a feeding RNAi strain carrying a fragment of the *daf*-16 (R13H8.1) coding sequence. These bacteria were also present throughout a 48 hour exposure to varying concentrations of cadmium chloride (0 to 22 µg ml^{-1}[= ppm] of Cd^{++}). The contents of each well were transferred to 96-well black non-fluorescent microplatea after 30 h (grey bars) and 48 h (black bars), and the level of GFP fluorescence measured in a Perkin-Elmer Victor 1420 fluorometric plate reader. Expression of a *daf*-16::GFP reporter was reduced by 50% using RNAi against DAF-16, as compared to the feeding vector only (data not shown). Details of the methodology used can be found in de Pomerai *et al.* (2003) [45].

stressors. Fleshing out our transgene-based SRN with genome-wide gene-array data [27-33] is also vital. We see our dynamic SRN model as a resource for the entire scientific community, and all we can hope to achieve after 3 years will be a first draft, to be updated and refined in the light of future findings so that its predictions become ever more accurate. The support and assistance received from colleagues will allow us to develop a well-rounded model that is more useful to everyone. A dedicated website for this project is now under development, through which we hope to make data summaries for all single-toxicant responses available in a read-only format to interested parties; this site will be accessible *via* links from the School of Biology website at the University of Nottingham (http://www.nottingham.ac.uk). Other scientists wishing to submit their own data (in a similar format) for inclusion in the process of SRN model development should initially make contact with the corresponding author (david.depomerai@nottingham.ac.uk).

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SUPPLEMENTARY MATERIAL

Supplementary material can be viewed at: http://www.bentham.org/open/totoxij.

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