Regulating resistance: CncC:Maf, antioxidant response elements and the overexpression of detoxification genes in insecticide resistance

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ABSTRACT

While genetic and genomic tools have greatly furthered our understanding of resistance-associated mutations in molecular target sites of insecticides, the genomic basis of transcriptional regulation of detoxification loci in insect pests and vectors remains relatively unexplored. Recent work using RNAi, reporter assays and comparative genomics are beginning to reveal the molecular architecture of this response, identifying critical transcription factors and their binding sites. Central to this is the insect ortholog of the mammalian transcription factor Nrf2, Cap 'n' Collar isoform-C (CncC) which as a heterodimer with Maf-S regulates the transcription of phase I, II and III detoxification loci in a range of insects with *CncC* knockdown or upregulation directly affecting phenotypic resistance. CncC:Maf binds to specific antioxidant response element sequences upstream of detoxification genes to initiate transcription. Recent work is now identifying these binding sites for resistance-associated loci and, coupled with genome sequence data and reporter assays, enabling identification of polymorphisms in the CncC:Maf binding site which regulate the insecticide resistance phenotype.

Exposure to insecticide instigates a complex response through which insects sequester, detoxify or excrete toxins before they reach their target or have other adverse consequences. The battery of detoxification genes and those elements which control their coordinated response has been labelled 'the defensome' [1]. The insect defensome must cope with a variety of assaults from foodstuffs e.g. haem breakdown products or plant allelochemicals, but has been latterly co-opted to deal with xenobiotic insecticidal challenge. Whilst the mammalian xenobiotic response has received much attention, a detailed understanding of the mechanistic basis of detoxifying enzyme upregulation in the insecticide resistance response of insects has been lacking. Recent work on both model, and non-model insects is beginning to redress this imbalance.

CncC:Maf regulates insecticide resistance and resistance-associated genes

Gene expression is regulated by a complex of transcriptional activators that bind to regions upstream of transcription start sites recruiting chromatin-modifying factors and the RNA polymerase II containing transcription initiation apparatus. Core RNA polymerase is capable of DNA dependant RNA synthesis *in vitro* but incapable of specific promoter recognition in the absence of additional factors. In eukaryotes a key transcriptional activator in the response to a wide variety of stressors is encoded by nuclear factor, erythroid 2-like *Nfe2l2* (*Nrf2*) [2-5], a mammalian bZIP family transcription factor that binds to specific promoter motifs – termed antioxidant response elements (AREs) - stimulating transcription. In mammals, Nrf2 is a key regulator of both developmental pathways and the rather nebulously titled 'stress response' [2-4]. Under normal conditions, Nrf2 is retained cytoplasmically, bound to the cytoskeletal ubiquitin ligase Keap1. Upon stress exposure, Nrf2 releases, translocates to the nucleus, and forms a heterodimer with a small Muscle Aponeurosis Fibromatosis (Maf-S) protein [6] binding to AREs upstream of a battery of antioxidant genes (Figure 1) including GSTs [7-9], carboxylesterases [10], cytochromes p450 [11] and ABC transporters [12] and is involved in regulation of the proteasome, serving to degrade damaged proteins and enzymes following stress-induced damage [13]. In *Drosophila* the insect *Nrf2* ortholog *Cap 'n' collar isoform C*, (*CncC*), is known to have

a central role in both development and the 'stress response' [5,14]. Xenobiotic exposure, including insecticidal challenge falls under this banner. If CncC:Maf regulates the expression of insecticide-resistance associated genes then perturbations to CncC levels, or ARE polymorphisms should alter both phenotypic insecticide resistance and detoxification gene expression. Thus, a regulatory role of CncC:Maf in the response to insecticides may occur through a variety of mechanisms: upregulation of *CncC*/*Maf* (leading to increased target transcription), down-regulation of *Keap1* (increasing nuclear translocation of CncC:Maf), mutations in key domains of these proteins, or mutations in AREs upstream of target genes affecting promoter activity. Metabolic insecticide resistance can occur due to either changes in enzyme activity resulting from coding polymorphisms or due to constitutive upregulation of detoxification genes. In either case, those transcription factors initiating expression of detoxification genes must themselves be constitutively expressed. *CncC* is itself constitutively activated in DDT-resistant *Drosophila* strains [15] as is *Nrf2* is in mammals [16] although these transcription factors do have a relatively short half-life (<20 min) [17]. Constitutive *CnnC* overexpression is also seen in a number of arthropods e.g. resistant *Tribolium* [18], *Anopheles stephensi* [1] and spider mites [19] suggesting that this may underlie the resistant phenotype in some instances. Although mutations to *CncC*, *Maf-S* or *Keap1* may have phenotypic effects, e.g. deletion of the NHB1 domain can result in induced expression of CncC targets [20] there is, as yet, no evidence that naturally occurring mutations to these highly conserved TFs underpin resistance.

Initial studies in *Drosophila* [21] demonstrated that either overexpressing *CncC*, or introducing a loss of function *Keap1* mutation not only upregulated the detoxifying enzyme *gstD1*, a gene with an upstream ARE, but also significantly increased survival to the toxic herbicide paraquat. By contrast, RNAi knockdown (KD) of *CncC* decreased both *gstD1* expression and survival demonstrating the importance of CncC:Maf for insect survival in the face of xenobiotic exposure. The first work to study the role of CncC:Maf in a true resistant phenotype used tissue specific *Keap1* KD (releasing CncC for cytoplasmic transposition) demonstrating a significant increase in resistance to the organophosphate malathion in *Drosophila melanogaster* [22]. The same study showed that >70% of genes upregulated

following phenobarbital (a prototypical inducer of the xenobiotic response) exposure are also upregulated by ectopic CncC exposure [22] demonstrating the breadth of effect of this TF. Recent work now shows the universality of the role of CncC:Maf in insecticide resistance with studies on *Drosophila*, flour beetles [18], Colorado potato beetles [23], *Aphis gosypii* [24] and spider mites (Arachnidae) [19] all showing that perturbing the CncC:Maf balance affects resistance to a variety of insecticides and alters the expression of key genes previously demonstrated to be involved in this resistance (Table 1). These studies have used a variety of approaches including *CncC*/*Maf* knockdown through RNAi, targeted GAL4/UAS overexpression of *CncC*/*Maf* and loss-of-function mutations in Keap1.

The decreasing cost of sequencing now enables understanding the whole transcriptomic response of perturbating CncC:Maf. In *Tribolium*, RNASeq analysis after *CncC* KD showed 662 genes had increased expression and 91 downregulation including a range of phase I, II and III genes [25]. It is unlikely that all have AREs and are under direct influence of CncC but that disturbing the CncC:Maf balance instigates a cascade response. Ingham *et al*. also knocked-down MAF in a multi-insecticide resistant strain of *Anopheles gambiae* [26]. KD increased mortality to DDT and pyrethroids (it did not redress full susceptibility but this strain is nearly fixed for target-site resistance mechanisms) and, through microarray analysis, the transcriptomic response to MAF KD was determined. Here, genes expressed differentially were correlated with a mined dataset of differentially expressed genes from multiple IR studies to identify transcripts upregulated in microarrays and correlated with CncC:Maf-S expression including the key Anopheline detoxification candidates *cyp6m2* and *Gstd1*.

Antioxidant Response Elements and Insecticide Resistance

Mammalian studies have identified a consensus ARE motif to which CncC:Maf binds: 5′-TMAnnRTGAYnnnGCRwwww-3′ [27]. The experimentally determined *Drosophila* motif is similar but whilst demonstrating a consensus exhibits substantial variability (Figure 2). This motif conservation enables its genome-wide identification computationally through positional matrix screening (see Fig

2) e.g. using Motifdb [28]. However, insects are a diverse and ancient Class (the time from the *Drosophila-Anopheles* MRCA is 265MY and *Drosophila-Myzus* 358MY *c.f.* 90MY between human and mouse) [29]. Since in mammalian systems a "universally applicable consensus sequence cannot be derived" [30], the presumption that the *Drosophila* positional matrix is appropriate for other insects remains untested. However, differences in *Tribolium* AREs [18] versus *Drosophila* (Figure 2) suggest AREs in other insects require experimental identification. The ideal method of identifying binding sites for CncC:Maf involves CHiP-Seq as undertaken in *Drosophila* [31-33]. A constraining factor on the ability to undertake ChIP-Seq for other insects is the lack of validated CncC or Maf antibodies (although ModEncode [34] circumvented such difficulties through use of ChIP-seq on transgenic flies expressing CncC-eGFP fusion proteins with immunoprecipitation performed using an anti-GFP antibody).

Both *in vivo* and *in vitro* reporter assays have been used to detect the functionality of AREs. Whilst such reporter assays clearly show AREs drive expression, in the absence of *CncC:Maf* overexpression, it is polymorphisms differentiating resistant from susceptible animals which will be causal of resistance and of use for resistance management [35]. Sometimes these may be gross polymorphisms. Inserted transposable elements (TEs) can carry TFBSs e.g. the Bari-Jheh TE brings new AREs upstream of two juvenile hormone epoxy hydrolase genes mediating survival to malathion and paraquat [36] and AREs are found in other *Drosophila* TEs [36]. SNPs are also a likely source. In humans, ARE sequence polymorphisms underlie inter-individual gene expression variation [27,37,38] with even single base changes affecting ARE functionality. Insects have much higher levels of sequence diversity than humans e.g. in *Anopheles* π=1.53% for a typical autosome within 1kbp upstream of genes where AREs would reside and across the genome there is 1 variant base every 2bp [39]. Thus it seems likely that ARE SNPs may affect expression and that there is a reservoir of SNPs in AREs which may be selected following insecticide challenge. Experimentally introduced ARE SNPs can be shown to affect detoxification gene expression e.g. mutagenesis of the ARE upstream of a *gstD1-GFP* reporter demonstrated only the WT ARE was inducible by stress (e.g. paraquat or H_20_2) indicating the effect of polymorphisms on promoter activity [21]. Kalsi and Palli [18] also examined reporter activity of various

CYP6B gene promoters from *Tribolium* demonstrating that SNPs can significantly affect expression. For *D. melanogaster* strains differing in DDT resistance levels a 15bp deletion in a CncC:Maf binding site exhibiting between-strain polymorphism correlated with DDT susceptibility [40] although when association studies of DDT resistance levels were conducted on the *Drosophila* Genetics Reference Panel, this variant was not associated with DDT resistance [41]. Whilst these studies demonstrate promoter activity of AREs, what is clearly needed is an understanding of the effect of ARE SNPs on resistance and expression e.g. using Crispr [42] driven disruption of AREs in defined genetic backgrounds.

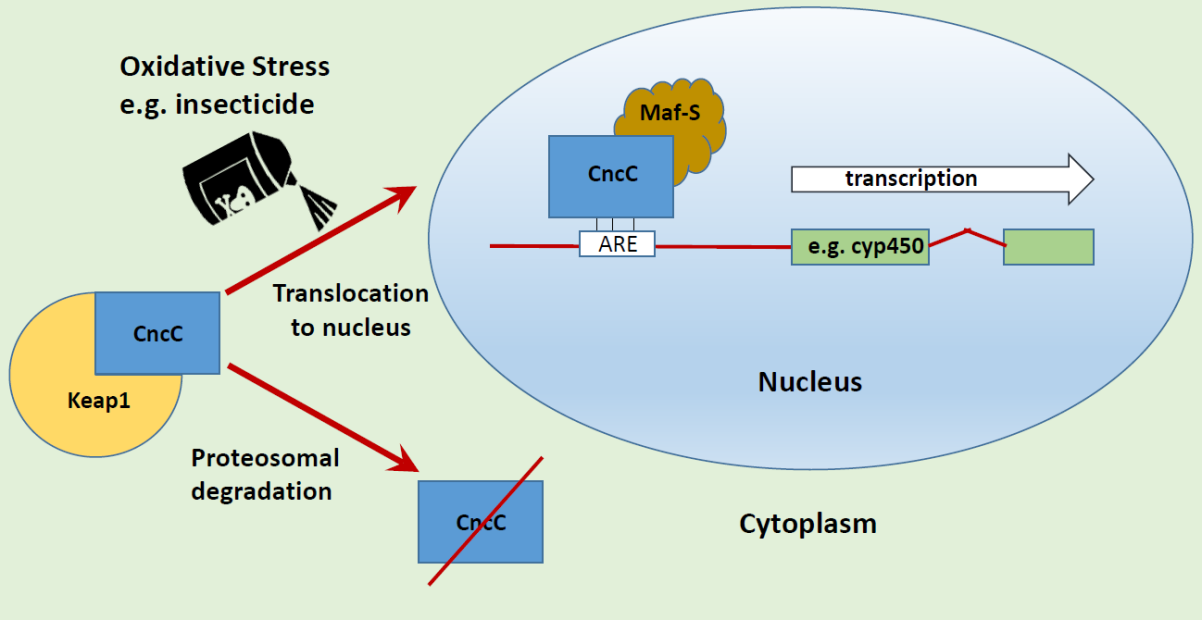
Role of other TFs in resistance

The transcription initiation machinery is complex and a CncC and ARE focus may be short-sighted. Kalsi and Palli [23] conducted RNAi knockdown studies in *Tribolium* on members of three superfamilies bHLH/PAS, bZIP and Nuclear Receptors (Table 1). KD of *CncC*, *Maf* or *Methoprene tolerant* all caused significant increases in mortality to the pyrethroid deltamethrin but crucially, only *CncC* and *Maf* KD also significantly altered the expression of key detoxification genes of the *Cyp6BQ* family. Whilst this appears to indicate the CncC:Maf pathway is more important in this phenotype, other transcription factors may be involved in other resistance phenotypes e.g. RNAi KD of the *Aphis gossypii* aryl hydrocarbon receptor affected the gossypol resistance associated *Cyp6AD2* [43], and reduced deltamethrin resistance in *T. castaneum* [18], the FOXA TF is implicated in *Bti* resistance in the Lepidopterans *Helicoverpa* and *Spodoptera* [44], and putative TF binding sites such as members of HNF family (also KD screened in [23]) have been identified in sequencing studies of resistant *Aedes* [45] and TFBSs identified in TEs inserted upstream of detoxification genes in *Drosophila* [46]. However, for these studies there has been no follow-up to identify and characterise their binding sites. This may be complicated since binding sites for other TFs may not be proximal (as are AREs) since upstream of genes lies both the proximal promoter and various *cis*-regulatory modules. The methods for identification and characterisation of TFBSs in CREs have been reviewed [47,48] and application of

153 these methods will address this knowledge gap. In *Drosophila* a large body of work is accumulating to
154 develop a comprehensive map of transcription factors and transcription factor binding sites (TFBSs)
155 [48-50] empowering computational approaches for TFBS identification e.g. [51]. Such work needs to
156 extend also into other insects given the economic and societal impacts of insecticide resistance. The
157 first step in this is knowledge of the TF repertoire and which genes are *cis*-regulated. Genome
158 sequencing efforts have enabled annotation of, for example, bHLH transcription factors in lice [52],
159 Psyliidae [53], *Nasonia* [54], *Nilaparvata* [55] and vector mosquitoes [56] and further work to
160 identification their roles and binding sites is necessary. As genome-wide allelic imbalance studies are
161 now demonstrably feasible and affordable for insects [57] identification of *cis*-regulated genes in
162 resistant insects will aid the honing of the search.

163 ***Conclusions and future directions***

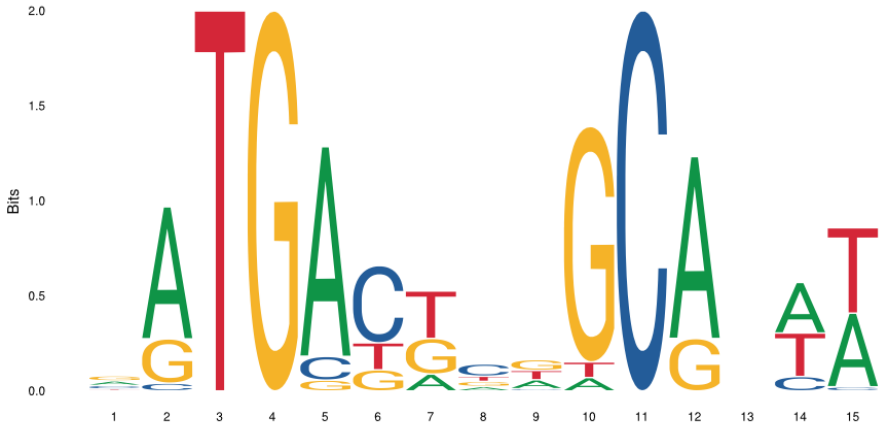
164 It is clear that CncC:Maf has an important role in insecticide resistance and that CncC upregulation
165 and/or polymorphisms in its response elements directly affect regulation of detoxification genes. The
166 high levels of phenotypic resistance seen in many insects to a range of insecticides cautions that other
167 transcription factors and enhancers are likely involved. The relative ease of study of CncC and its
168 proximal ARE should not draw attention away from searching for other TFs and characterising these
169 in the way that has started to occur for CncC:Maf. Concerted efforts employing comparative genomics,
170 true GWAS, CHiP-Seq and Crispr to further our understanding of this complex phenotype is needed.



171

172 Figure 1. Under normal conditions CncC is held in the cytoplasm by the ubiquitin ligase Keap1 and
173 degraded through the proteasome pathway. Under oxidative stress such as insecticidal exposure,
174 CncC dissociates from Keap1, translocates to the nucleus and forms a heterodimer with Maf-S. The
175 CncC/Maf heterodimer binds to antioxidant response elements (AREs) upstream of target genes and
176 initiates transcription, in the example here of a cytochrome P450.

A.



B.

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CYP6BQ1  ATGCCGCAGTGCCGATG
CYP6BQ6  ACCGTGCAGTAAGTTTG
CYP6BQ7  ATCCAGCAGTAGAAGGG
CYP6BQ8  ACCGTGCAGTAAGTTTG
CYP6BQ9  ACGGTGCAGTTCATGTG
CYP6BQ10 ATACTGCAGTTGCAATT
CYP6BQ10 CTGATGCAGTACTCACG
CYP6BQ12 TGATTGCAGTACGAGAT
CYP6BQ12 ATTATGCAGTTGGAAAA
CYP6BQ12 GAAATGCAGTTTTTATC
CYP6BQ12 ACACGGCAGTGCTTGTT
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Figure 2. Variability in the antioxidant response element sequence. 2A. Sequence logo for CncC:Maf-S ARE binding site in *Drosophila melanogaster* identified through ChIP-seq experiments. Logo generated at jaspar.genereg.net (Matrix ID: MA0530.1) [58]. Figure 2B. Alignment of AREs identified upstream of key cytochrome P450 genes of insecticide resistant *Tribolium castaneum* [18]. Note that whereas the sequence logo for *Drosophila* indicates a high likelihood for a C at position 11, at the equivalent position in the *Tribolium* AREs is a T (boxed). Note that Position 1 in Figure 2A = base five of the mammalian ARE (5′-TMAnnRTGAYnnnGCRwwww-3′)

Table 1. RNAi knockdown of transcription factors involved in insecticide resistance in insect and arachnid species. *CncC = cap 'n' collar isoform C*, *Ahr = aryl hydrocarbon receptor*, *Arnt = Aryl Hydrocarbon Receptor Nuclear Translocator*, *Maf-S = small Muscle Aponeurosis Fibromatosis*, *Met = Methoprene tolerant*, *HNF4 = Hepatocyte Nuclear Factor 4*, *HR96 = hormone receptor-like in 96*, *Spineless = aryl hydrocarbon receptor analog*, *USP = Ultraspiracle* (*Retinoid X receptor* homolog which heterodimerizes with the ecdysone receptor regulating ecdysone response genes). Since CncC must form a functional heterodimer with MAF it is unclear whether in this heterodimer CncC or Maf-S are the most appropriate KD target. MAF can homodimerize and it is possible that it engages other targets in this form, whilst CncC operates only as part of a heterodimer, however KD of either gene appears to cause phenotypic effects with parallel KDs often affecting the expression of the same genes.

Species	Phenotype	KD target	Effect on phenotypic resistance	Effect on gene expression	Reference
Hemiptera					
Aphis gossypii	gossypol tolerance	*CncC*	Increased gossypol tolerance	*Cyp6AD2* downregulated (qPCR)	[24]
Aphis gossypii	gossypol tolerance	*Ahr*, *Arnt*	Increased gossypol tolerance	*Cyp6AD2* downregulated (qPCR)	[43]
Coleoptera					
Tribolium castaneum	Deltamethrin resistance	*CncC* *Maf-S* *Met* *HNF4* *HR96* *Spineless* *USP*	Increased mortality Increased mortality Increased mortality No significant effect No significant effect No significant effect No significant effect	*CncC* KD: *Cyp6BQ2*, *Cyp6BQ4*, *Cyp6BQ6*, *Cyp6BQ7*, *Cyp6BQ9*, *Cyp6BQ11*, *Cyp6BQ12* (qPCR) *MAF: Cyp6BQ2*, *Cyp6BQ3*, *Cyp6BQ4*, *Cyp6BQ5*, *Cyp6BQ6*, *Cyp6BQ7*, *Cyp6BQ9*, *Cyp6BQ10*, *Cyp6BQ12* (qPCR)	[18]
Tribolium castaneum	Deltamethrin resistance	*CncC*	Not tested, but see above	662 genes upregulated, 91 downregulated (RNASeq). *CnCC*, *Cyp6BQ2*, *Cyp6BQ6*, *Cyp6BQ7*, *Cyp6BQ9* (qPCR)	[25]
Leptinotarsa decemlineata	Imidacloprid resistance	*CncC*	Survival decreased from 54% to 5% following KD	*Cyp9Z25*, *Cyp9Z29*, *Cyp6BJ1v1*, *Cyp6BJ*$^{a/b}$	[23]
Lepidoptera					
Helicoverpa armigera	*Bti* resistance (Cry1AC toxin)	*Fox-A*	Lower *Bti* mortality and higher pupation following KD	*ABCC2*, *ABCC3* (qPCR)	[44]

Diptera					
Anopheles gambiae	Permethrin, deltamethrin, DDT resistance	*Maf-S*	Increased mortality to DDT, permethrin, deltamethrin. No effect on bendiocarb mortality. Decreased mortality to malathion	Reduced expression of *Cyp6M2*, *GstD1*, *GstD3 Jheh1*, *Jheh2*, *Gnmt*. Increased expression of *Cyp4H17*	[26]
Culex quinquefasciatus	Permethrin resistance	*GSαS Adenylyl cyclase*	Increased permethrin susceptibility	*GSαS* KD: *Cyp9M10*, *Cyp6AA7*, *Cyp9J34* (qPCR) *AC KD*: *Cyp9M10*, *Cyp9J34*, *Cyp9J40, Cyp6AA7* (qPCR)	[59]
Drosophila melanogaster	Paraquat survival	*CncC* *Keap1*	Decreased paraquat survival	*gstD1* expression reduced *gstD1* expression increased	[21]
Drosophila melanogaster		*CncC* *Keap1*	 Increased malathion resistance	Reduced expression of *Cyp6a2*, *Cyp6a8*, *gstD2*, *gstD7*, *Jheh1* (qPCR)	[22]
Drosophila melanogaster	DDT resistance	*CncC*		Reduced expression of *Cyp6a2*, *Cyp6a8* (qPCR)	[15]
Acari					
Tetranychus cinnabarinus	Fenpropathrin resistance	*CncC* *Maf-S*	LC_{30} increased from 12.75% to 19.5%	CncC KD: decreased expression of Cyp389B1, Cyp391A1, Cyp392A28. MAF KD: Cyp389B1, Cyp392A28.	[19]

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*15. Insecticide resistance mediated through elevated expression of detoxification genes is a constitutive rather than an induced phenomenon. Misra *et al*. show that *CncC* is constitutively expressed in resistant strains of *Drosophila* and that this constitutively expressed gene causes upregulation of key detoxification genes.

**18. Kalsi and Palli knocked-down a variety of transcription factors and demonstrated that it is CncC/MAF that controls upregulation of the CYP6BQ genes, previously implicated in pyrethroid resistance in flour beetles but also that ARE elements in the CYP6BQ promoter promote expression in reporter assays co-transfected with CncC and Maf.

*21. An older but comprehensive study of the role of *CncC* in *Drosophila*. A molecular biology *tour de force* employing a variety of methods to show how *CncC* is involved in detoxification and aging.

*25. Following injection of dsRNA (CncC or GFP) RNASeq was used by Kalsi and Palli to understand the role of CncC in the transcriptomic response in insecticide resistant *Tribolium*. This is the only study to use RNASeq to study the role of CncC/Maf.

**26. Ingham *et al*. use RNAi knockdown of *Maf-S* in the Tiassalé strain of *Anopheles gambiae* followed by whole-genome microarrays to identify genes regulated by CnCC/Maf. They then compare the differentially regulated genes to those genes identified as differentially expressed across a number of transcriptomic studies of the insecticide resistance phenotype in mosquitoes.

*37. Although not a study of the insects or insecticide resistance, Kuosmanen *et al*. utilised a variety of approaches (molecular modelling, analysis of CHiP datasets and protein binding microarrays) to show how sequence variation in AREs can affect NRF2 binding and be associated with disease

resistance. Such work is now needed for the insecticide resistance phenotype in insect genomic databases.

**47. This excellent and comprehensive review covers experimental and computational approaches for identifying regulatory motifs in genomes. It focuses on more distal *cis*-regulatory elements which are likely to be more problematical to identify than proximal AREs. Application of these methods to insect species beyond *Drosophila* may identify other TFs (other than CncC) and their binding sites involved in the insecticide resistance phenotype.