Gene co-expression network reveals highly conserved, well-regulated anti-ageing mechanisms in old ant queens.

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Abstract

Evolutionary theories of ageing predict a reduction in selection efficiency with age, a so-called 'selection shadow', due to extrinsic mortality decreasing effective population size with age. Classic symptoms of ageing include a deterioration in transcriptional regulation and protein homeostasis. Understanding how ant queens defy the trade-off between fecundity and lifespan remains a major challenge for the evolutionary theory of ageing. It has often been discussed that the low extrinsic mortality of ant queens, that are generally well protected within the nest by workers and soldiers, should reduce the selection shadow acting on old queens. We tested this by comparing strength of selection acting on genes upregulated in young and old queens of the ant, *Cardiocondyla obscurior*. In support of a reduced selection shadow, we find old-biased genes to be under strong purifying selection. We also analysed a gene co-expression network (GCN) with the aim to detect signs of ageing in the form of deteriorating regulation and proteostasis. We find no evidence for ageing. In fact, we detect higher connectivity in old queens indicating increased transcriptional regulation with age. Within the GCN, we discover five highly correlated modules that are upregulated with age. These old-biased modules regulate several anti-ageing mechanisms such as maintenance of proteostasis, transcriptional regulation and stress response. We observe stronger purifying selection on central hub genes of these old-biased modules compared to young-biased modules. These results indicate a lack of transcriptional ageing in old *C. obscurior* queens possibly facilitated by strong selection at old age and well-regulated anti-ageing mechanisms.

Introduction

Ageing, the progressive decline of physiological function with age, and thus of survival and fertility, is common to most multicellular species (Jones et al., 2014). Extensive genetic and molecular studies have illuminated several proximate mechanisms involved in the ageing process, allowing us to better understand *how* we age. The majority of these "hallmarks of ageing" can be attributed to the accumulation of cellular damage (López-Otín et al., 2013; Gems and Partridge, 2013) and an overall deterioration of regulation (Frenk and Houseley, 2018). One important hallmark of ageing, the loss of protein homeostasis, is caused by a reduction in quality control mechanisms such as chaperones that support correct folding and structure of proteins, as well as proteolytic pathways that ensure the removal of misfolded peptides (Koga et al., 2011; Rubinsztein et al., 2011; Tomaru et al., 2012; Calderwood et al., 2009; López-Otín et al., 2013). The result is an accumulation of toxic, misfolded proteins and an inefficient replenishment of correctly functioning proteins. Further hallmarks of ageing include deleterious changes in terms of cell-cycle (a cessation of cellular replication), intercellular communication, nutrient sensing and epigenetic regulation (López-Otín et al., 2013), as well as a downregulation of mitochondrial and protein synthesis genes (Frenk and Houseley, 2018). Importantly, the ageing process is often accompanied by a dysregulation of

transcription (Frenk and Houseley, 2018).

Several classic evolutionary theories of ageing aim to explain *why* organisms age (Kirkwood and Austad, 2000; Flatt and Partridge, 2018). These theories generally describe a reduction in selection efficiency with increasing age because the number of surviving individuals decreases due to extrinsic mortality. In the mutation accumulation theory, this 'selection shadow' leads to an accumulation of mutations which have a deleterious effect later in life (Kirkwood and Austad, 2000; Flatt and Partridge, 2018). Building on this, the antagonistic pleiotropy theory describes how genes with beneficial effects early in life can be maintained by selection even if they have pleiotropic negative effects later in life (Williams, 1957). In the disposable soma theory, the pleiotropic effect of more specific genes is described, that cause a trade-off between somatic maintenance and reproduction (Kirkwood, 1977), so that an increased, or early, investment in offspring is expected to come at the price of a shorter lifespan and vice versa (Kirkwood and Austad, 2000).

There are, however, exceptions to these expectations; possibly most notably within social insects, where reproductive castes exhibit relatively long lifespans compared to their sterile siblings (Keller and Genoud, 1997). This apparent lack of a trade-off between longevity and fecundity in social insects is at odds with expectations for the disposable soma theory. The longer life of queens compared to sterile castes might be explained by low extrinsic mortality due to the protection of a well-defended nest (Keller and Genoud, 1997; Negroni et al., 2016). The low extrinsic mortality of queens can in turn be expected to lead to a reduction of the selection shadow as more queens reach old-age, allowing efficient selection on genes that are important for somatic maintenance late in life.

In an attempt to understand the relationship between fecundity and longevity in social insects, several studies have investigated caste and age-specific expression of putative ageing genes in honeybees (Aamodt, 2009; Aurori et al., 2014; Corona et al., 2005, 2007; Seehuus et al., 2013), ants (Lucas et al., 2016; Lucas and Keller, 2018; Negroni et al., 2019a; Von Wyschetzki et al., 2015) and termites (Kuhn et al., 2019; Elsner et al., 2018). One of these studies, which compared gene expression between young and old queens of the ant *Cardiocondyla obscurior*, identified several overlaps with ageing pathways known from *Drosophila melanogaster* (Von Wyschetzki et al., 2015). However, surprisingly, for many genes the ratio of expression level between old and young ant queens was reversed compared to *D. melanogaster*. Further studies comparing expression between castes and age-groups highlight the importance of several gene pathways for longevity in social insects that have previously been implicated in ageing, such as antioxidants (Aurori et al., 2014; Corona et al., 2005; Negroni et al., 2019b; Kuhn et al., 2019), immunity (Negroni et al., 2019b; Aurori et al., 2014; Lucas and Keller, 2018; Kuhn et al., 2019; Negroni et al., 2016), DNA and somatic repair (Kuhn et al., 2019; Aamodt, 2009; Lucas et al., 2016; Seehuus et al., 2013), respiration (Lockett et al., 2016; Corona et al., 2005), as well as the insulin/insulin-like growth

factor (IGF) signaling (IIS) (Kuhn et al., 2019; Aurori et al., 2014) and the target of rapamycin (TOR) signalling pathways (Negroni et al., 2019b; Kuhn et al., 2019). The IIS and TOR nutrient sensing pathways are of particular interest in this context, since their role in longevity and fecundity has been extensively studied in model organisms (Tatar et al., 2003; Partridge et al., 2011; Kenyon, 2010; Flatt and Partridge, 2018). These transcriptional studies offer insights into individual genes and their pathways that might be involved in ageing in social insects. However, a more holistic view of gene networks is likely to uncover further important genes as well as insights into transcriptional regulation. For example, a study of gene co-expression networks on mouse brains revealed that with age a decrease in the correlation of expression between genes occurred, showing that transcriptional dysregulation can lead to a significant reduction in gene connectivity (Southworth et al., 2009). These findings demonstrate the application of transcriptional studies for investigating whole pathways and gene networks and their wide-reaching implications for ageing. Furthermore, the extent at which a selection shadow may be reduced for old queens due to a reduction in extrinsic mortality has so far not been formally tested.

To address these questions we investigated transcriptomic data available for young and old queens of the polygynous ant, *C. obscurior* (Von Wyschetzki et al., 2015). These ant queens are relatively short-lived compared to most ant species (median lifespan: 16-26 weeks Kramer et al. 2015; Schrempf et al. 2005), which is in accordance with expectations for polygynous species, where extrinsic mortality is higher than in monogynous colonies (Keller and Genoud, 1997). Nevertheless, as for most ant species, *C. obscurior* queens (up to 48 weeks) outlive sterile workers that are expected to live around 12 to 16 weeks (Oettler and Schrempf, 2016). Importantly, consistently high reproductive output throughout their lives until immediately before death indicates no apparent reproductive senescence in these ant queens (Kramer et al., 2015). To test for signs of ageing in transcriptional regulation, we carried out a gene co-expression network analysis, in which we identified gene modules related to young mated (4 weeks) and old mated (18 weeks) queens and compared overall network connectivity. We also tested the hypothesis that, due to low extrinsic mortality, selection efficiency should not decline with age in queens. We found evidence for an array of anti-ageing mechanisms that are more tightly regulated in old queens. We could find no evidence for a selection shadow, indicating stable selection efficiency throughout an ant queen life.

Results and Discussion

Old-biased genes are not under weaker selection

Evolutionary theories of ageing predict weaker selection on genes which are expressed in old individuals due to low effective population size and reduced fecundity (Kirkwood and Austad, 2000; Flatt and

Partridge, 2018). In ant queens, we may expect a reduction of this 'selection shadow' as low extrinsic mortality and lifelong, high fertility should lead to a stable effective population size up to old age. We tested this by comparing evolutionary rates between three groups of genes. These were (i) old-biased genes: significantly over-expressed in seven old (18 weeks) compared to seven young (4 weeks) *C. obscurior* queens; (ii) young-biased genes: significantly over-expressed in young compared to old queens; (iii) unbiased genes: no significant difference in expression between young and old queens. To estimate evolutionary rates and thus infer direction and strength of selection, we measured dN/dS (rate of nonsynonymous to synonymous substitution rates) for one-to-one orthologs with a set of 10 ant species (see methods). A dN/dS ratio ≈ 1 indicates neutral evolution, whereas values $<< 1$ signify purifying selection. We find no evidence for weaker purifying selection in old-aged queens, since dN/dS in old-biased genes (median: 0.084) is in fact significantly lower than in young-biased genes (median: 0.127; p-value = 0.016; Mann-Whitney U test; fig. 1), indicating increased purifying selection with age. Interestingly, dN/dS in young-biased genes is also significantly lower than in unbiased genes (median: 0.100; p-value = $2.2\text{x}10^{-}4$; Mann-Whitney U test), as has previously been reported for the ant, *Lasius niger* (Lucas et al., 2017). This is in contrast to published results for age-biased genes in humans, in which old-biased genes had a significantly higher dN/dS (median: 0.22) than young-biased (median: 0.09, p = $1.4\text{x}10^{-50}$), as would be expected for a reduction in purifying selection with age (Jia et al., 2018). To further test the ability of this method to detect a selection shadow in insects, we repeated the analysis for *D. melanogaster*. Age-biased gene expression was measured for a novel data set containing expression data for young (10 days) and old (38 days) female flies across two tissues (head and fat body) and different feeding regimes. Evolutionary rates were obtained for these genes from published analyses based on alignments of 12 *Drosophila* species (Consortium et al., 2007). In contrast to our results for ant queens but in agreement with expectations for a selection shadow, we find significantly higher dN/dS levels in old-biased fly genes (median: 0.060) compared to young-biased genes (median: 0.047; p=$5.1\text{x}10^{-}8$; Mann-Whintey U test).

We also investigated the numbers of ant genes that are under significant positive selection within old-biased compared to young-biased and unbiased genes, using a site test of the codeml suite (Yang, 1997). Contrary to expectations for weaker selection strength on old queens, we found no difference in the proportion of genes under positive selection between the three groups of genes (old-biased: 21.7%; young-biased: 21.9%; unbiased: 16.0%; $\text{Chi}^2 = 3.3$; p = 0.19). The effect size of the observed difference in proportions of genes under positive selection between young- and old-biased genes is so low (cohen's h: 0.003), that we assume the lack of significance is not due to a lack of power. The genes under significant positive selection in old-biased genes contain two regulatory genes (transcription factor and methyltransferase), an electron transport protein, a member of the COPI coatomer complex (important for protein transport) and *Notch* (Table 1). The latter is the central signalling protein within the Notch

signalling pathway which is involved in tissue homeostasis and age-related diseases (Balistreri et al., 2016).

Contrary to expectations based on evolutionary theories of ageing, these results suggest selection is not weaker on genes expressed mainly in old queens. We speculate that high fertility in old queens, coupled with an overall low extrinsic mortality, which is typical for social insects (Negroni et al., 2016; Keller and Genoud, 1997), may reduce the selection shadow in *C. obscurior* queens, leading to similar selection strength throughout their fertile life.

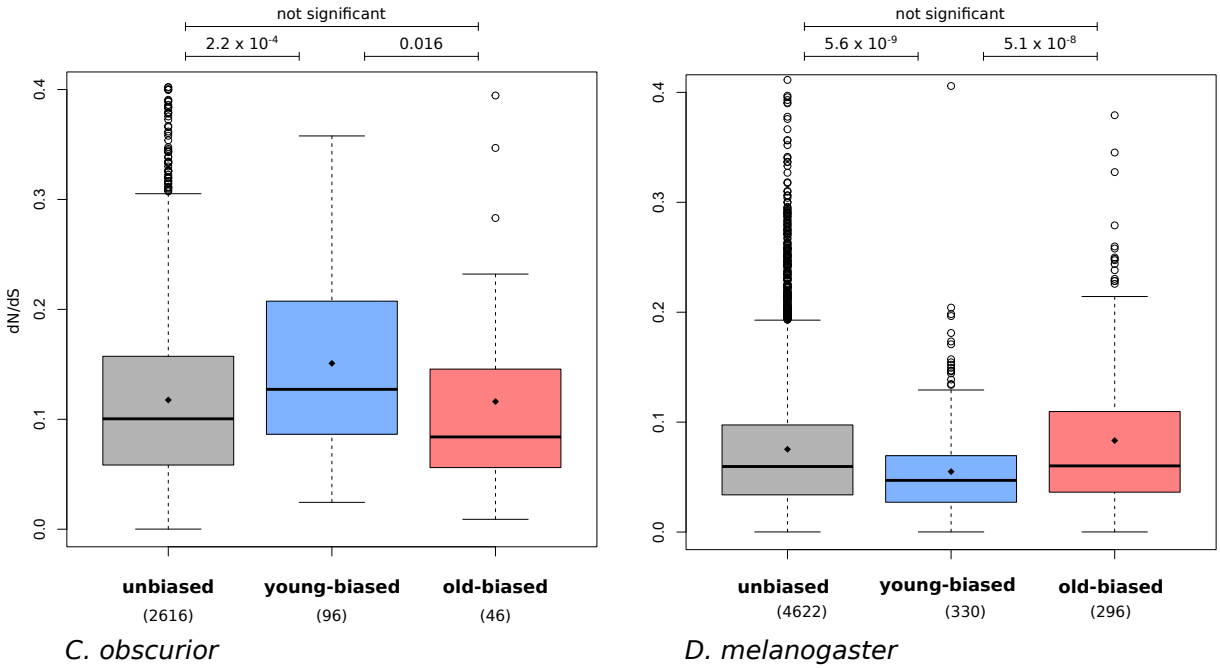


Figure 1: Evolutionary rates (dN/dS) in genes with unbiased expression, young-biased and old-biased expression in *C. obscurior* queens and *D. melanogaster* adult females.
Significance was tested with Mann-Whitney U test.

Table 1: Old-biased genes under significant positive selection.

Gene	Ortholog	Putative Function
Cobs_01221	uncharacterised	unknown
Cobs_04278	FBgn0002121 (l(2)gl)	polarity of neuroblasts and oocytes
Cobs_06663	FBgn0085424 (nub)	transcription factor
Cobs_08231	FBgn0004647 (Notch)	tissue homeostasis
Cobs_08620	FBgn0027607 (Dymeclin)	organisation of Golgi apparatus
Cobs_09212	FBgn0033686 (Hen1)	methyltransferase, methylates siRNA & piRNA
Cobs_11651	FBgn0036714 (CG7692)	unknown function
Cobs_12452	FBgn0008635 (βCOP)	subunit of the COPI coatomer complex, transport from Golgi to ER
Cobs_16420	FBgn0034745 (CG4329)	unknown
Cobs_16765	Cytochrome b561 domain-containing protein 1 (Q8N8Q1)	electron transport protein

Increased connectivity in old ant queens

In old queens, we expected to find little evidence for age-related transcriptional dysregulation in the form of reduced correlation of gene expression, as previously reported for ageing mouse brains (Southworth et al., 2009). We investigated this by measuring gene connectivity separately within old queens and within young queens, using the *softConnectivity* function of the WGCNA package (Langfelder and Horvath, 2008). This connectivity describes the total strength of correlations that a gene possesses with all other genes in a gene co-expression network (GCN; Langfelder and Horvath 2008) and is thought to correlate positively with gene essentiality (Carlson et al., 2006). In fact, we find gene expression connectivity to be significantly higher in older queens (median: 145.3) than within young queens (median: 142.5; effect size: 0.255; $p = 4.3\text{x}10^{-29}$; Wilcoxon signed-rank test), suggesting an increased regulation of gene networks in older queens.

The more highly connected genes in older queens (1471 genes with connectivity fold change > 2) are enriched for GO term functions (FDR < 0.1) related to protein synthesis, transcription, purine synthesis, cellular respiration and ATP metabolism (Table S1). Most of the 20 genes with the strongest increase in connectivity in old queens (4.8-7.1 fold increase) compared to young queens are involved in transcriptional regulation (7 genes) or protein homeostasis (6 genes; Table S2). For example, a member of the 26S proteasome complex, important for the degradation of misfolded proteins, is the gene with the highest increase in connectivity in old queens. As has been shown for several organisms, including humans (Lee et al., 2010), yeast (Kruegel et al., 2011) and *C. elegans* (Vilchez et al., 2012), increased proteosome activity can extend lifespan by reducing proteotoxic stress (López-Otín et al., 2013). An increase in connectivity of fatty-acid synthase 3 may have implications for colony communication. Further highly

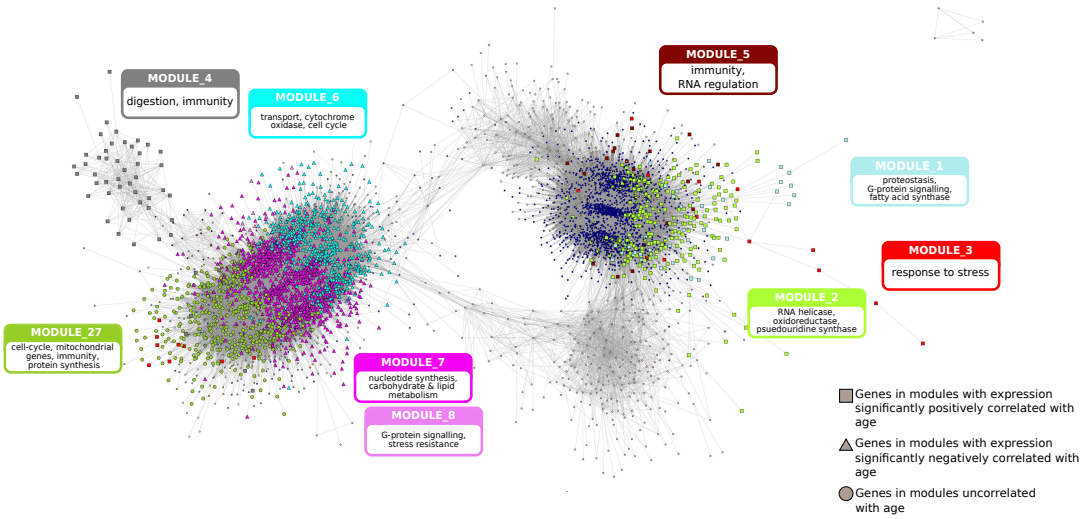
connected genes include ribosomal proteins or genes involved in the correct folding, post-translation modification or transport of proteins. The genes with highly increased connectivity in old ant queens, which are involved in transcriptional regulation, include two transcription factors, a transcritional co-regulator (*taranis*), and four mRNA regulators. These results suggest that, contrary to expectations for ageing individuals, increased transcriptional regulation and protein homeostasis takes place in old queens.

Co-expression modules related to age

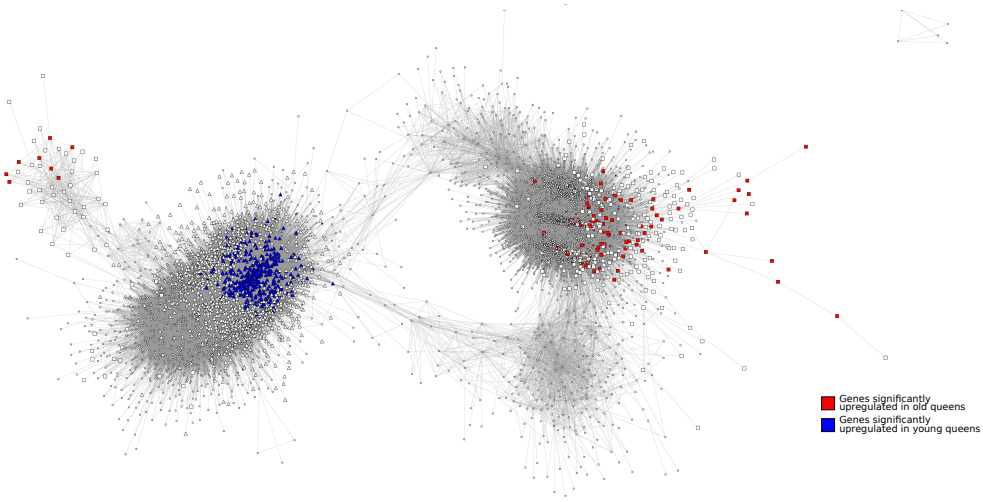
We constructed a signed, weighted gene co-expression network (GCN, Langfelder and Horvath 2008) based on the correlation of normalised gene expression across all 14 samples (7 young queens & 7 old queens). Within the GCN, genes could be grouped into 27 modules, within which gene expression was especially strongly correlated (Fig. 2). To determine the importance of these modules for old and young queens, we first calculated eigengene expression based on the first principal component of each module. We then correlated eigengene expression of each module with the binary trait 'age' (young & old). Five of the modules were significantly, positively correlated with age ($p < 0.05$), indicating an overall higher expression of these modules in old compared to young queens. Three modules were significantly, negatively correlated with young queens, indicating a downregulation in old queens. To validate these correlations, we analysed difference in expression of genes between old and young queens ($\log_2[\text{expression}_{old}/\text{expression}_{young}]$) within each of these modules. Accordingly, the median $\log_2$-fold-change in expression was greater than zero in each of the old-biased modules (0.148 to 0.340) and less than zero within the young-biased modules (-0.376 to -0.249; Fig. S1). Four of the five old-biased modules (1, 2, 3 and 5) belonged to a larger cluster within the GCN, which is quite distant from the cluster containing the young-biased modules (6, 7, 8; Fig. 2). module_4 (old-biased), on the other hand, forms a more distinct cluster, adjacent to the young-biased cluster. The old-biased modules contained several genes that had previously been identified as upregulated in old queens via standard differential expression analysis (Von Wyschetzki et al., 2015) but contained no genes that were upregulated in young queens. The opposite was true for young-biased modules, thus confirming the validity and compatibility of both methods (Fig. 2(b)).

However, importantly, the GCN analysis also allowed the identification of many additional age-related genes that can not be identified by standard differential expression analyses. For example, module_1, which has the strongest association with old queens ($\rho = 0.96$; $p = 5.3\text{x}10^{-8}$; Pearson correlation), contains 109 genes, of which only 41 are individually significantly differentially expressed between old and young queens. Similarly, module_6, which is strongly negatively associated with old queens ($\rho r = -0.90$; $p = 9.4\text{x}10^{-6}$; Pearson correlation), contains 970 genes, of which 240 were identified as individually

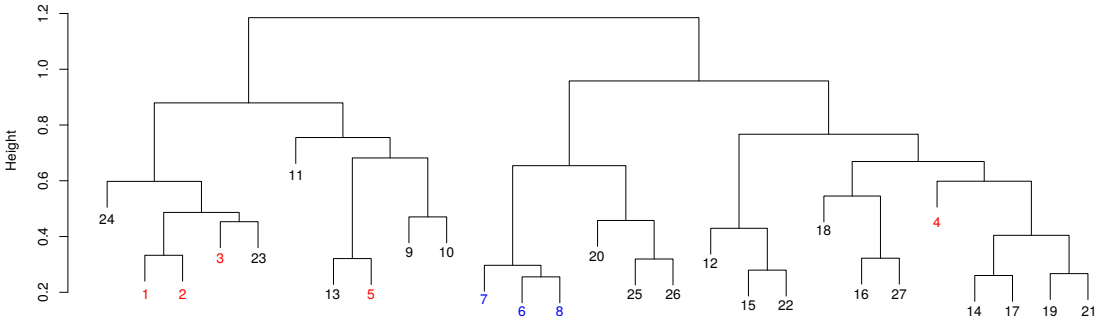
significantly upregulated in young queens (Von Wyschetzki et al., 2015). In the following section, we describe these eight age-biased modules in terms of their functional enrichment and detail the top hub genes (genes with the highest intramodular connectivity) within these modules.



(a) GCN modules



(b) Differentially expressed genes



(c) Clustering of modules

Figure 2: Caption on next page.

Figure 2: (Previous page.) Gene co-expression network (GCN).
(a & b) Graphical representation of the gene co-expression network, containing only the most strongly connected genes (n = 5442). In (a) genes are coloured according to the modules to which they belong. The main enriched functions (based on hubs and GO terms) of the 9 discussed modules are labelled (see text for more details). In (b) genes are coloured according to their differential expression; red: over-expressed in old queens; blue: over-expressed in young queens; white: not differentially expressed. In both representations, genes in modules significantly related to old queen expression are depicted as squares, and those significantly related to young queens are triangles; all other genes are represented by circles.(c) Clustering dendogram of modules; height represents dissimilarity based on topological overlap. Modules significantly related to age are highlighted in red (positive correlation) and blue (negative correlation).
Higher resolution image available in the online version.

Old-biased modules

The most highly connected hub genes in ***module_1***, the module most strongly upregulated with age ($\rho = 0.96$; p = 5.3x$^{10-8}$; 109 genes; Fig. 3), include three genes with functions related to maintaining and restoring proteostasis in old queens (Table S3), the loss of which has been described as one of the hallmarks of ageing (López-Otín et al., 2013). These are: a member of the TRAPP complex, important for protein transport, *Socs44A*, a gene involved in ubiquitination and *GRXCR1*, responsible for the post-transcriptional S-glutathionylation of proteins, a modification which is often triggered as a defence against oxidative stress (Dalle-Donne et al., 2009). The top hubs of this module also include two genes which encode integral members of the G-protein signalling pathway, namely, a Rho guanine nucleotide exchange factor and a G-protein α-subunit. The most connected gene within this hub is a fatty-acid synthase which may play an important role in colony communication. This module is enriched for a GO term related to the regulation of transcription (Table S4).

Module_2 (596 genes;upregulated with age: $\rho = 0.65$; p = 0.012) contains hub genes coding for proteins with diverse functions, including an RNA helicase, a maternal protein, a protein with oxidoreductase activity and a pseudouridine synthase (Table S3).

Module_3 (433 genes; upregulated with age; $\rho = 0.63$; p = 0.017) is particularly interesting since it is not only upregulated with age but, on average, gene members of the module are more strongly connected within old than in young queens (Fig. 3). Hub genes indicate this module is important for responses to age-related stress, especially processes related to a maintenance of proteostasis (Table S3). For instance, the top 10 hubs contain the endoplasmic reticulum (ER) stress protein, disulfide-isomerase, which reacts to protein misfolding and oxidative stress (Laurindo et al., 2012), as well as *fringe*, which modulates Notch signalling, a pathway important for regulating tissue homeostasis and implicated in ageing related diseases (Balistreri et al., 2016). A further hub is a trehalose transporter, orthologous to *tret1-2*, indicating that the transport of trehalose (the main haemolymph sugar in insects) from fat body to other tissues is well regulated in old queens (Kanamori et al., 2010). This may have a positive effect

on survival, since trehalose treatment increases longevity in *C. elegans* (Honda et al., 2010).

The top 10 hub genes in ***module_4*** (186 genes; $\rho = 0.61$; p = 0.021) fulfil various functions, such as the digestive enzymes alpha glucosidase and chymotrypsin-1, indicating a possible modification in diet with age (Table S3). The third most connected gene within this module is orthologous to *pirk* in *D. melanogaster* (involved in the negative regulation of the immune response; Kleino et al. 2008), indicating the immune system may be downregulated with age in *C. obscurior*. Interestingly, long-lived flies also tend to downregulate the induction of immune effector genes (Fabian et al., 2018; Loch et al., 2017). This module is enriched for the GO term transmembrane transport (Table S4).

Module_5 (169 genes; $\rho = 0.58$; p = 0.028) may be important for controlling the immune system since two hub genes (Table S3), the COMM domain containing protein 8 (COMMD8) and the WD40 domain containing angio-associated migratory cell protein (AAMP), are both known to inhibit the transcription factor NF-κ-B (Burstein et al., 2005; Bielig et al., 2009). An upregulation of NF-κ-B occurs with ageing and its inhibition, as apparently occurs within this module, can reduce the effects of senescence (Tilstra et al., 2012). Interestingly, COMMD8 is also characterised by a strong increase in connectivity (1.68 fold change), indicating its heightened importance in old queens. Further functions of this module may be related to RNA regulation, as evidenced by the hub gene *eyes_absent*, a transcription factor with importance in embryonal eye development in *D. melanogaster* (Bonini et al., 1998). Based on the ten nearest neighbours in the *C. obscurior* GCN, *eyes_absent* may regulate several enzymes involved in post-transcriptional processes, such as mRNA export from the nucleus (*sbr*, Cobs_03187), and tRNA modification (*Tgt*: Cobs_16650; *HisRS*: Cobs_01013; CG3808: Cobs_18201).

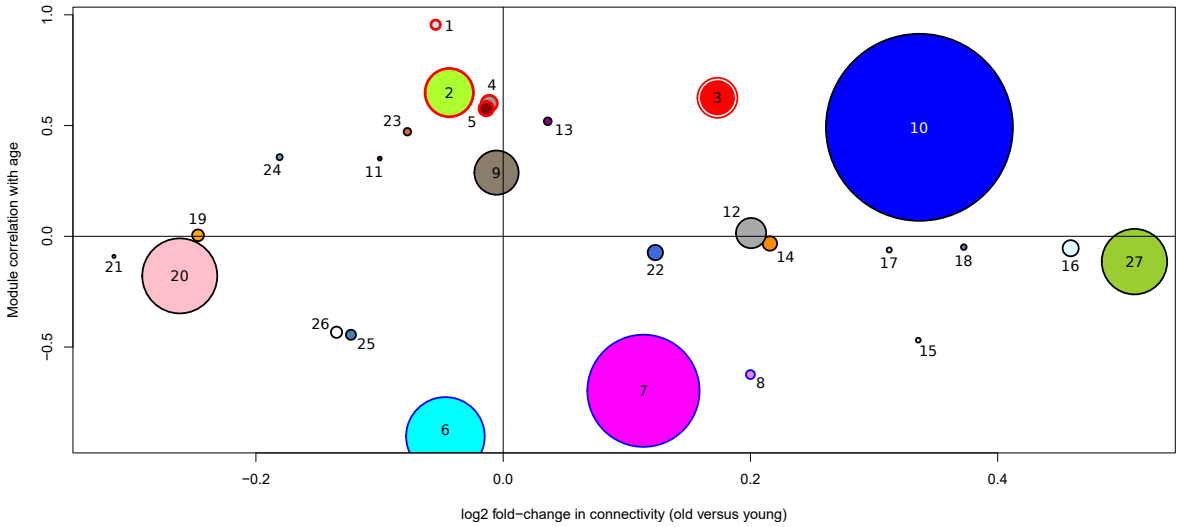


Figure 3: Correlation of GCN modules with age and their change in connectivity between old and young queens. A positive correlation with age (y-axis) signifies an upregulation of a module in old queens. A positive log2foldchange in connectivity (x-axis) represents a higher connectivity in old queens. Modules are labelled with their assigned module numbers. Sizes of dots represent relative number of genes within modules. Modules with red outlines are significantly upregulated and modules with blue outlines are significantly downregulated in old queens compared to young queens.

Modules downregulated with age

Module_6 (970 genes) is the module most strongly down-regulated with age (ρ = -0.9; p = 9.4x10^{-6}) and is enriched for the GO terms transmembrane transport and potassium ion transport (Table S4). Interestingly, the top 10 hubs contain three genes with no detectable homology to any protein in the uniprot arthropod database (Table S3). Otherwise, the functions of hub genes in this module span various functions, such as cell-cell interactions, cytochrome oxidase, an odorant receptor and a negative regulator of the cell cycle.

Module_7 (1385 genes; ρ = -0.7; p = 0.006) has several enriched functions in the nucleotide synthetic process, oxidoreductase activity, carbohydrate and lipid metabolism, ATP metabolic processes, cofactor and coenzyme binding (Table S4). Accordingly the top hubs in this module contain a thioredoxin, a proteasome subunit (α6) and two genes involved in ubiquitination (*STUB1* and *Ubc6*; Table S3).

Module_8 (103 genes; ρ = -0.62; p = 0.08) is enriched forthe function G-protein coupled receptor activity (Table S4). The top hub gene in this module (intraconectivity 0.90), Cobs_08138, is orthologous to the *methuselah-like receptors* in *D. melanogaster* (Friedrich and Jones, 2016). Interestingly, mutant flies, carrying P-element insertions in one of these *methuselah* genes, live 35% longer and are significantly

more resistant to stresses than wild-types (Lin et al., 1998). There are indications that these effects on lifespan and stress response may represent the ancestral function of methuselah receptors in *Drosophila* (Araújo et al., 2013). A similar function of the *methuselah* ortholog in *C. obscurior* would explain how a reduction in expression within older queens may facilitate life extension and greater stress resistance.

We also examined ***module_27*** (808 genes) in more detail since it shows the strongest increase in connectivity in old compared to young queens (1.47 fold) of all modules (Fig. 3), suggesting an increased regulation of this module with age. The functions connected to this module, based on hubs (Table S3), increases in connectivity (Table S5) and GO terms (Table S4), indicate that in old queens an increased regulation of cell-cycle, mitochondrial genes, immunity genes, transcriptional genes and members of the protein synthesis machinery takes place, which is in stark contrast to the expected gene expression hallmarks of ageing in multicellular eukaryotes (Frenk and Houseley, 2018).

Old-biased module hubs are highly conserved

We investigated evolutionary rates of the most connected genes within the old- and young-biased modules. Hub genes (intraconnectivity $> 50\%$) of the five old-biased modules have significantly lower rates of protein evolution (dN/dS median: 0.081) than hubs in young-biased modules (median: 0.118; $p = 6.0\text{x}10^{-4}$) or compared to all lowly connected genes (intraconnectivity $< 50\%$; median: 0.101; $p = 0.017$). These results suggest the hub genes of old-biased modules are highly constrained by strong purifying selection.

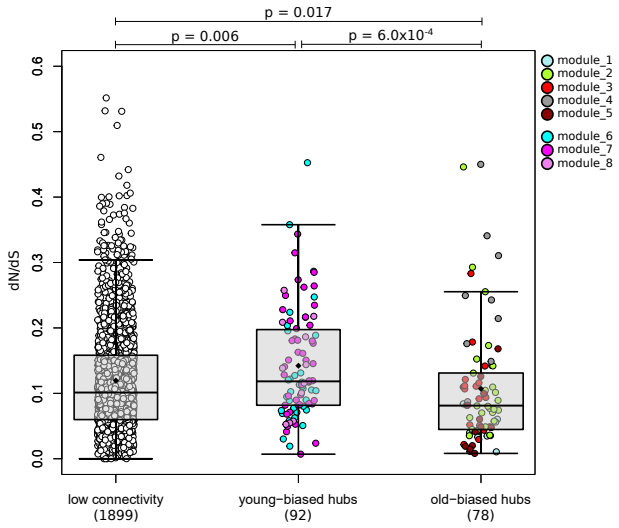


Figure 4: dN/dS rates in hub genes within young- and old-biased modules compared to lowly connected genes. Each dot represents a gene, which are coloured by the module membership. Whiskers of the boxplots represent up to 1.5 times the interquartile range. Black diamonds are means, and horizontal bars within the boxes are medians.
Hub genes have an intraconnectivity > 50%; lowly connected: < 50%.

IIS and TOR pathways

The role of the IIS and TOR pathways in both ageing and fertility is well documented (e.g. Tatar et al. 2003; Partridge et al. 2011; Flatt and Partridge 2018). An increase in activity of the IIS and TOR pathways, generally through an uptake of carbohydrates and amino acids, respectively, is linked to an increase in fecundity and a reduction in lifespan. As this pattern is apparently altered in *C. obscurior*, where differences in fecundity do not affect lifespan (Schrempf et al., 2017, 2005), we compared these two intertwined pathways between young and old queens (Fig. 5).

Interestingly, the two central transcription factors *foxo* and *tor* in old queens had significant correlation to two and six other genes, respectively, in old queens but no significant correlations in young queens. A greater connectivity of these important transcription factors in old queens may improve functionality thus contributing to sustained high reproduction. We extracted expression data for these genes from the aforementioned novel data set, which compared fat body gene expression between old and young adult females in *D. melanogaster*. In contrast to the ant queen data set, the connectivity of *tor* was reduced in old flies (3 to 1), while the connectivity of *foxo* was relatively stable (3 in young flies & 4 connections in old flies).

One major difference in individual connections in the comparison between species could be observed for *rheb*, a positive regulator of the TOR protein in *D. melanogaster* (Strilbytska et al., 2017). In terms

of gene expression, *rheb* is negatively correlated with the *tor* gene in old *C. obscurior* queens (r = -0.456, p = 0.023), which is also negative but non-significant in young queens (r = -0.333, p = 0.46), indicating a possible inhibitory effect on *tor*. The known positive regulation of the TOR protein by Rheb in *D. melanogaster* does not necessarily mean that expression levels of *tor* and *rheb* should be positively correlated. However, we do find a significant positive correlation in gene expression between *rheb* and *tor* in old flies (r = 0.296, p = 0.037). A reversal of this correlation in ant queens suggests an alternative relationship exists between *rheb* and *tor* in *C. obscurior*, which becomes stronger in older individuals. If indeed, the Rheb protein has an inhibitory effect on TOR in *C. obscurior*, this could have implications for the causal effect of nutrition on longevity and fertility, which may be linked to the high reproductive output of queens even at high age.

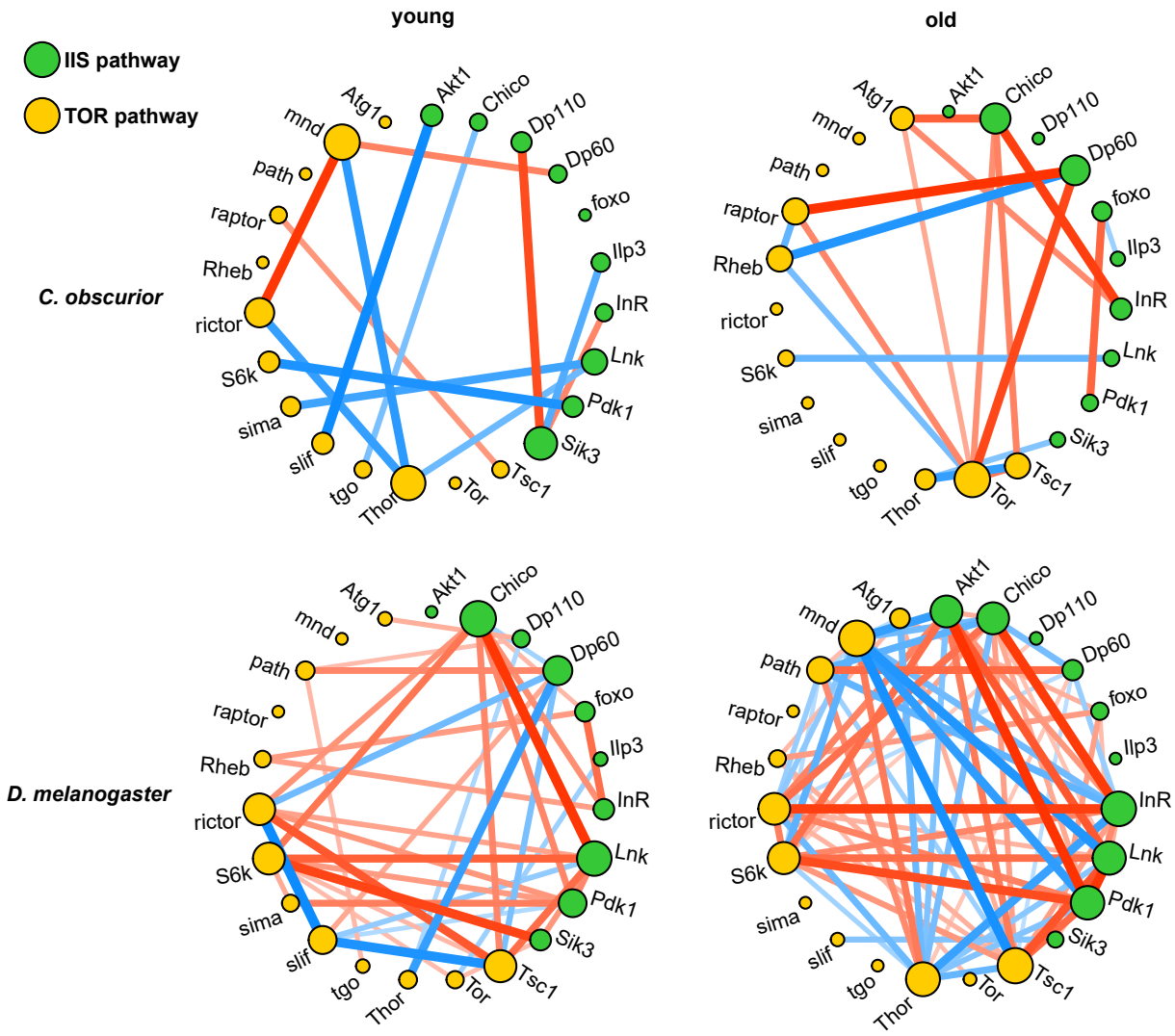


Figure 5: Correlations of expression between 23 core genes within the IIS and TOR pathways in young and old *C. obscurior* queens, compared to *D. melanogaster*.
Red lines represent positive and blue lines negative correlations. The strength of correlations is represented by the thickness of lines, size of nodes is relative to their connectivity. Only significant correlations are shown ($p < 0.05$). Members of the TOR pathway are yellow, members of the IIS pathway are green.

Conclusions

Evolutionary theory of ageing predicts a selection shadow on genes expressed late in life due to a reduction in effective population size with increasing age caused by extrinsic mortality (Kirkwood and Austad, 2000). We expected to find a reduced selection shadow in *C. obscurior* queens, as ant queens generally experience low extrinsic mortality. In support, we find compelling evidence for strong purifying selection on old-biased genes (significantly upregulated in 7 old compared to 7 young queens), for which evolu-

tionary rates (dN/dS) are significantly lower than young-biased genes. In contrast, we find evidence of a selection shadow in *D. melanogaster* where dN/dS is significantly higher for old-biased genes. Our results suggest, therefore, that *C. obscurior* queens are not affected by a selection shadow, so that genes important at old age can not be expected to accumulate deleterious mutations at an increased rate compared to early-acting genes. This offers an explanation for the apparent lack of ageing and the high reproductive output of old ant queens.

Furthermore, we were interested in understanding whether *C. obscurior* queens show signs of ageing, especially within transcriptional regulation. This is a particularly intriguing question since the reproductive fitness of these ant queens remains high until old age, although they outlive their sterile siblings (Oettler and Schrempf, 2016). In fact, our analysis of co-expression networks in *C. obscurior* queens uncovers a significant increase in gene connectivity in old queens. This result offers evidence for an increased transcriptional regulation, especially in genes that are themselves involved in transcriptional regulation,as well as several genes involved in protein synthesis and degradation, which are important mechanisms for counteracting symptoms of ageing (Frenk and Houseley, 2018). Also, the analysis of old-biased modules (clusters of highly correlated genes, upregulated with age) within the GCN revealed an increase in expression and connectivity of genes involved in proteostasis, stress response, and transcriptional regulation (Fig. 2(a)), offering further support for well-regulated anti-ageing mechanisms. The hub genes within these old-biased modules are more highly conserved than hubs of young-biased modules, indicating strong purifying selection acting on these important central regulators.

We also found tentative evidence for a rewiring of the IIS and TOR pathways, which have far reaching effects on both longevity and fecundity (Tatar et al., 2003; Partridge et al., 2011; Flatt and Partridge, 2018). In these pathways we find an increase in connectivity of the central transcription factors, *tor* and *foxo* in old compared to young queens. The relationship between *tor* and its direct upstream regulator, *rheb*, is reversed in *C. obscurior* queens compared to *D. melanogaster*.

In summary, we find no evidence of ageing in transcriptional regulation in *C. obscurior* queens. Low extrinsic mortality may allow selection to shape genes important at old age, which is evident in low divergence rates (dN/dS) of the hubs of old-biased modules. Well regulated molecular mechanisms likely allow the ant queens to counteract any symptoms of ageing, thus maintaining high reproductive fitness throughout life. We suggest further transcriptional studies into the short period directly before death when the reproductive output of *C. obscurior* queens decreases (Heinze and Schrempf, 2012; Kramer et al., 2015), which we expect to illuminate processes of transcriptional ageing. Transcriptional studies of other ant species are necessary to investigate the generality of our findings. In monogynous ants, for example, in which individual queens are less dispensible, we would expect to observe an even weaker selection shadow. Also, *C. obscurior* queens are relatively short-lived compared to other ant species. Selection strength on

age-biased genes of extremely long-lived queens may be less affected by reductions in effective population sizes due to longer generation times. Further detailed research on individual pathways is important to understand how an upregulation of anti-ageing mechanisms occurs; especially proteomic analyses may reveal the true relationships between pathway members. The IIS and TOR pathways are an important starting point for such studies in this and other social insect species, where we have found indications for a rewiring that may explain the absence of the fecundity/longevity trade-off.

Methods

Data set

Genome and proteome sequences of the *C. obscurior* genome, version 1.4, were obtained from the hymenopteragenome.org website (accessed July 2018; Elsik et al. 2015). We estimated gene functions based on orthology, primarily to *D. melanogaster*, as well as PFAM domains and GO terms. Putative protein functions were based on descriptions in the flybase (Thurmond et al., 2018) and UniProt (Consortium, 2018) databases, unless otherwise stated. We calculated orthology to *D. melanogaster* with the method of reciprocal best blast hit (Rivera et al., 1998). For this, the proteomes of *C. obscurior* and *D. melanogaster* (v. 6.21; obtained from `ftp://ftp.flybase.net/releases/current/dmel_r6.21/fasta/`; accessed June 2018) were blasted against each other using blastp (BLAST 2.7.1+; Camacho et al. 2009) and an e-value threshold of $1e^{-5}$. Reciprocal best blast hits were extracted from the output files using a custom perl script. Where no orthology could be detected using this method, protein sequences were blasted against the swissprot database with blastp (version 2.7.1+; Altschul et al. 1990) and the best hit was retained with an evalue < 0.05. Protein sequences were annotated with PFAM domains using pfamscan (Mistry et al., 2007), to which GO terms were mapped with pfam2GO (Mitchell et al., 2014).

Published RNAseq data were obtained for 7 old (18 weeks) and 7 young (4 weeks) ant queens from NCBI (Von Wyschetzki et al., 2015). These queens had each been individually reared from pupal stage in separate experimental colonies, each containing 20 workers and 10 larvae, originating from the genome reference population in Bahia, Brazil (Von Wyschetzki et al., 2015; Schrader et al., 2014). Fastq files were mapped to the *C. obscurior* genome (version 1.4) with hisat2 (Kim et al., 2019) using default parameters. We then indexed and sorted sam files using samtools (version 1.7, Li et al. 2009) and generated counts per gene using htseq-count (Anders et al., 2015). All statistical analyses on these counts were carried out in R (version 3.5.1, R Core Team 2018). Where necessary, we corrected for multiple testing with the `p.adjust` function, using the fdr method (Benjamini and Hochberg, 1995). A total of 10 339 genes were expressed in at least two individuals with a read count of at least 10. This subset of genes was used for all analyses.

Determining age-biased expression

Within this subset of 10 339 genes, we identified genes with age-biased expression by comparing expression in the 7 old to the 7 young samples. This was carried out with the R package DESeq2 at default settings (Love et al., 2014). Genes with an adjusted p-value < 0.05 were deemed either old- or young-biased. All other genes were classified as unbiased.

Molecular evolution and selection analyses

In order to carry out evolutionary analyses, we first determined orthology between the proteomes of *C. obscurior* and 9 further ant species, which we either downloaded from the hymenopteragenome.org website (accessed August 2020; Elsik et al. 2015): *Atta cephalotes*, *Pogonomyrmex barbatus*, *Solonopsis invicta* and *Wasmannia auropunctata*; or NCBI (accessed August 2020): *Monomorium pharaonis*, *Temnothorax cuvispinosus*, *Temnothorax longispinosus*, *Vollenhovia emery*. Data for *Crematogaster levior* were obtained from the authors of the genome publication upon request (Hartke et al., 2019). Orthology was determined with OrthoFinder (Emms and Kelly, 2015) at default settings. We chose orthologous groups that contained single gene copies within each of the 10 species. Protein sequences of each ortholog set were aligned with prank (version 170427, Löytynoja 2014) at default settings. The corresponding CDS sequences were aligned using pal2nal (Suyama et al., 2006). CDS alignments were trimmed for poorly aligned codon positions with Gblocks (version 0.91b) with the following parameters: -t=c -b2=6 -b3=100000 -b4=1 -b5=h. We calculated dN/dS ratios using the null model of codeml in the PAML suite (Yang, 1997), using the following tree based on a published ant phylogeny (Ward et al., 2015):

(((((((Tlon,Tcur),Clev),Veme),Cobs),(Waur,Acep)),(Mpha,Sinv)),Pbar)

dN/dS rates were used for analyses only if dS < 3. dN/dS rates were compared between old-biased, young-biased and unbiased genes using the Mann-Whitney test with the R function *wilcox.test*.

In order to detect genes that contain codon sites under positive selection, we performed a likelihood-ratio test (LRT) between models 7 (null hypothesis; dN/dS limited between 0 and 1) and 8 (alternative hypothesis; additional parameter allows dN/dS > 1) of the codeml program within the PAML suite (Yang, 1997). For this we used runmode 0, model 0 and set 'NSsites' to 7 & 8.

Gene co-expression analysis

The expression counts data were normalised using the built-in median of ratios method implemented by default in DESeq2 (version 1.22.2, Love et al. 2014) and then transposed to a matrix containing genes in columns and samples in rows. With the reduced set of 10 339 genes, we created a signed

weighted gene co-expression network using the WGCNA package (version 1.68, Langfelder and Horvath 2008) that incorporated expression values from all 14 queen samples (7 young and 7 old). We followed the standard stepwise protocol (`https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/`), using a soft power of 14 and the biweight midcorrelation function for calculating coexpression similarity. Minimum module size was set at 30 and resulting modules with a correlation of at least 0.75 were merged. Hub genes within modules were determined based on the intra-modular connectivity, which we calculated with the `intramodularConnectivity` function on the adjacency matrix, that was produced during the WGCNA pipeline.

To compare connectivity between young and old queens, we calculated connectivity with the `softConnectivity` function separately within the young and the old queen expression data. We used the same soft power value of 14 and the biweight midcorrelation function.

To create a visualisation of the GCN, the topological overlap matrix was reduced to only contain genes with a topological overlap of at least 0.1 to at least one other gene. Edge and node files were created with the WGCNA function `exportNetworkToCytoscape`, using a threshold of 0.1. All further visualisations of the network were conducted in Cytoscape (v. 3.7.2, Shannon et al. 2003).

For the analysis of the IIS and TOR pathways, we calculated pairwise correlations of all pathway members using the `bicorAndPvalue` function of the WGCNA package. Only correlations with a p-value < 0.05 were considered in this study.

GO enrichment

GO term enrichment analyses were carried out with topGO (version 2.34.0; Alexa and Rahnenfuhrer 2018) on the "biological process" category, using the classic algorithm. Node size was set to 5, Fisher statistic was implemented and we only kept GO terms that matched at least 3 genes and with a p-value $<$ 0.05. An FDR was added using the R function p.adjust and the method "fdr" (Benjamini and Hochberg, 1995).

D. melanogaster data set for analysis of IIS/TOR pathways

To compare the age-related changes in correlations within the IIS/TOR pathways we found for *C. obscurior* with *D. melanogaster*, we accessed a recently compiled, but so far unpublished, RNAseq data set (SRA accession: PRJNA615318). This data set comprised RNAseq of 34 samples of 5 pooled flies. We used y^1,w^{1118} mutant flies (full genotype: yw; +/+; +/+). These flies were maintained in laboratory conditions at 25°C, 12h:12h light:dark and 60% relative humidity.

Experimental setup

Adult virgin females and males were collected separately, and 3 days later they were pooled together to freely mate. Eggs were laid in a controlled density (50-100 eggs per bottle) and developed until the adult stage in the same conditions as mentioned above. After eclosion, the offspring adult flies matured for one day. On the second day after eclosion, female and male flies were collected and transfered to a demographic cage. Each cage contained 130 females and 70 males. Once cages were set up, they were divided into four groups, which consisted of 4 different diet treatments. The diet treatments differed only in the content of yeast (20, 40, 80 or 120g) present in the fly food; the other ingredients were added in the same quantities in all diets (1L water, 7g agar, 50g sugar, 10mL 20% nipagin and 6mL propionic acid). All cages were maintained in the same conditions as described above.

Sampling and RNA extractions

Female flies were sampled at two time points: 10 days (young) and 38 days (old). For each time point, sampling and dissections were done between 1 pm and 6 pm. Two groups of 5 females each (2 replicates) were anesthetized in the fridge (approximately 4°C), and afterwards fat bodies were dissected in ice-cold 1x PBS. To guarantee that we sampled the entire fat body, we decided to use in this experiment fat bodies still attached to the cuticle – usually referred to as fat body enriched samples – because the cuticle is transcriptionally inactive. In ice-cold PBS, the female fly abdomens were opened, and the organs were carefully removed. Once the fat body tissue was clean, the abdomen cuticle was separated from the thorax. The fat body enriched tissues were transferred into Eppendorf with 200µL of homogenization buffer from the RNA isolation kit (MagMAX™-96 Total RNA Isolation Kit from Thermo Fisher). The tissues were homogenized and stored at -80°C until RNA extraction. To sample head transcriptomes, flies were transferred to Eppendorfs and snap-frozen with liquid nitrogen. Then the Eppendorfs were vigorously shaken to separate the heads from the bodies. The heads were then transferred into an Eppendorf containing 200µL of homogenization buffer, from the RNA isolation kit. As described above, tissues were homogenized in the solution and kept at -80°C until RNA extraction. All extractions were done using the MagMax robot from Thermo Fisher and the MagMAX™-96 Total RNA Isolation Kit. In this experiment there is a total of 34 samples: 2 time points X 4 diet treatments x 2 tissues = 16 groups, for each group we have 2-3 replicates (all groups have 2 replicates except for the second time point for 2% yeast diet, where we have 3 replicates). The sequencing of the RNA samples was done in BGI, Hong Kong, China. The samples were sequenced (paired-end, 100bp) on an Illumina HiSeq 4000 platform. Gene counts were generated in the same manner as for *C. obscurior* using genome version 6.21 (obtained from `ftp://ftp.flybase.net/releases/current/dmel_r6.21/fasta/`; accessed June 2018).

References

Aamodt, R. M. (2009). Age-and caste-dependent decrease in expression of genes maintaining dna and rna quality and mitochondrial integrity in the honeybee wing muscle. *Experimental Gerontology*, 44(9):586–593.

Alexa, A. and Rahnenfuhrer, J. (2018). *topGO: Enrichment Analysis for Gene Ontology*. R package version 2.34.0.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *Journal of molecular biology*, 215(3):403–410.

Anders, S., Pyl, P. T., and Huber, W. (2015). Htseq—a python framework to work with high-throughput sequencing data. *Bioinformatics*, 31(2):166–169.

Araújo, A. R., Reis, M., Rocha, H., Aguiar, B., Morales-Hojas, R., Macedo-Ribeiro, S., Fonseca, N. A., Reboiro-Jato, D., Reboiro-Jato, M., Fdez-Riverola, F., et al. (2013). The drosophila melanogaster methuselah gene: a novel gene with ancient functions. *PloS one*, 8(5):e63747.

Aurori, C. M., Buttstedt, A., Dezmirean, D. S., Mărghitaş, L. A., Moritz, R. F., and Erler, S. (2014). What is the main driver of ageing in long-lived winter honeybees: antioxidant enzymes, innate immunity, or vitellogenin? *Journals of Gerontology Series A: Biomedical Sciences and Medical Sciences*, 69(6):633–639.

Balistreri, C. R., Madonna, R., Melino, G., and Caruso, C. (2016). The emerging role of notch pathway in ageing: focus on the related mechanisms in age-related diseases. *Ageing research reviews*, 29:50–65.

Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal statistical society: series B (Methodological)*, 57(1):289–300.

Bielig, H., Zurek, B., Kutsch, A., Menning, M., Philpott, D., Sansonetti, P., and Kufer, T. (2009). A function for aamp in nod2-mediated nf-κb activation. *Molecular immunology*, 46(13):2647–2654.

Bonini, N. M., Leiserson, W. M., and Benzer, S. (1998). Multiple roles of theeyes absentgene indrosophila. *Developmental biology*, 196(1):42–57.

Burstein, E., Hoberg, J. E., Wilkinson, A. S., Rumble, J. M., Csomos, R. A., Komarck, C. M., Maine, G. N., Wilkinson, J. C., Mayo, M. W., and Duckett, C. S. (2005). Commd proteins, a novel family of structural and functional homologs of murr1. *Journal of Biological Chemistry*, 280(23):22222–22232.

Calderwood, S. K., Murshid, A., and Prince, T. (2009). The shock of aging: molecular chaperones and the heat shock response in longevity and aging–a mini-review. *Gerontology*, 55(5):550–558.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden, T. L. (2009). Blast+: architecture and applications. *BMC bioinformatics*, 10(1):421.

Carlson, M. R., Zhang, B., Fang, Z., Mischel, P. S., Horvath, S., and Nelson, S. F. (2006). Gene connectivity, function, and sequence conservation: predictions from modular yeast co-expression networks. *BMC genomics*, 7(1):40.

Consortium, D. . G. et al. (2007). Evolution of genes and genomes on the drosophila phylogeny. *Nature*, 450(7167):203.

Consortium, U. (2018). Uniprot: a worldwide hub of protein knowledge. *Nucleic acids research*, 47(D1):D506–D515.

Corona, M., Hughes, K. A., Weaver, D. B., and Robinson, G. E. (2005). Gene expression patterns associated with queen honey bee longevity. *Mechanisms of ageing and development*, 126(11):1230–1238.

Corona, M., Velarde, R. A., Remolina, S., Moran-Lauter, A., Wang, Y., Hughes, K. A., and Robinson, G. E. (2007). Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *Proceedings of the National Academy of Sciences*, 104(17):7128–7133.

Dalle-Donne, I., Rossi, R., Colombo, G., Giustarini, D., and Milzani, A. (2009). Protein s-glutathionylation: a regulatory device from bacteria to humans. *Trends in biochemical sciences*, 34(2):85–96.

Elsik, C. G., Tayal, A., Diesh, C. M., Unni, D. R., Emery, M. L., Nguyen, H. N., and Hagen, D. E. (2015). Hymenoptera genome database: integrating genome annotations in hymenopteramine. *Nucleic acids research*, 44(D1):D793–D800.

Elsner, D., Meusemann, K., and Korb, J. (2018). Longevity and transposon defense, the case of termite reproductives. *Proceedings of the National Academy of Sciences*, 115(21):5504–5509.

Emms, D. M. and Kelly, S. (2015). Orthofinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. *Genome biology*, 16(1):157.

Fabian, D. K., Garschall, K., Klepsatel, P., Kapun, M., Lemaitre, B., Schl, C., Arking, R., and Flatt, T. (2018). Evolution of longevity improves immunity in Drosophila. *Evolution Letters*, 00(0):1–13.

Flatt, T. and Partridge, L. (2018). Horizons in the evolution of aging. *BMC biology*, 16(1):1–13.

Frenk, S. and Houseley, J. (2018). Gene expression hallmarks of cellular ageing. *Biogerontology*, 19(6):547–566.

Friedrich, M. and Jones, J. W. (2016). Gene ages, nomenclatures, and functional diversification of the methuselah/methuselah-like gpcr family in drosophila and tribolium. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 326(8):453–463.

Gems, D. and Partridge, L. (2013). Genetics of longevity in model organisms: debates and paradigm shifts. *Annual review of physiology*, 75:621–644.

Hartke, J., Schell, T., Jongepier, E., Schmidt, H., Sprenger, P. P., Paule, J., Bornberg-Bauer, E., Schmitt, T., Menzel, F., Pfenninger, M., et al. (2019). Hybrid genome assembly of a neotropical mutualistic ant. *Genome biology and evolution*, 11(8):2306–2311.

Heinze, J. and Schrempf, A. (2012). Terminal investment: individual reproduction of ant queens increases with age. *PLoS One*, 7(4).

Honda, Y., Tanaka, M., and Honda, S. (2010). Trehalose extends longevity in the nematode caenorhabditis elegans. *Aging cell*, 9(4):558–569.

Jia, K., Cui, C., Gao, Y., Zhou, Y., and Cui, Q. (2018). An analysis of aging-related genes derived from the genotype-tissue expression project (gtex). *Cell death discovery*, 4(1):1–14.

Jones, O. R., Scheuerlein, A., Salguero-Gómez, R., Camarda, C. G., Schaible, R., Casper, B. B., Dahlgren, J. P., Ehrlén, J., García, M. B., Menges, E. S., et al. (2014). Diversity of ageing across the tree of life. *Nature*, 505(7482):169.

Kanamori, Y., Saito, A., Hagiwara-Komoda, Y., Tanaka, D., Mitsumasu, K., Kikuta, S., Watanabe, M., Cornette, R., Kikawada, T., and Okuda, T. (2010). The trehalose transporter 1 gene sequence is conserved in insects and encodes proteins with different kinetic properties involved in trehalose import into peripheral tissues. *Insect biochemistry and molecular biology*, 40(1):30–37.

Keller, L. and Genoud, M. (1997). Extraordinary lifespans in ants : a test of evolutionary theories of ageing. *Nature*, 389(October):3–5.

Kenyon, C. J. (2010). The genetics of ageing. *Nature*, 464(7288):504.

Kim, D., Paggi, J. M., Park, C., Bennett, C., and Salzberg, S. L. (2019). Graph-based genome alignment and genotyping with hisat2 and hisat-genotype. *Nature biotechnology*, 37(8):907–915.

Kirkwood, T. B. (1977). Evolution of ageing. *Nature*, 270(5635):301–304.

Kirkwood, T. B. and Austad, S. N. (2000). Why do we age? *Nature*, 408(6809):233.

Kleino, A., Myllymaki, H., Kallio, J., Vanha-aho, L.-M., Oksanen, K., Ulvila, J., Hultmark, D., Valanne, S., and Ramet, M. (2008). Pirk Is a Negative Regulator of the Drosophila Imd Pathway. *The Journal of Immunology*, 180(8):5413–5422.

Koga, H., Kaushik, S., and Cuervo, A. M. (2011). Protein homeostasis and aging: The importance of exquisite quality control. *Ageing research reviews*, 10(2):205–215.

Kramer, B. H., Schrempf, A., Scheuerlein, A., and Heinze, J. (2015). Ant colonies do not trade-off reproduction against maintenance. *PLoS One*, 10(9):e0137969.

Kruegel, U., Robison, B., Dange, T., Kahlert, G., Delaney, J. R., Kotireddy, S., Tsuchiya, M., Tsuchiyama, S., Murakami, C. J., Schleit, J., et al. (2011). Elevated proteasome capacity extends replicative lifespan in saccharomyces cerevisiae. *PLoS genetics*, 7(9):e1002253.

Kuhn, J. M. M., Meusemann, K., and Korb, J. (2019). Long live the queen, the king and the commoner? transcript expression differences between old and young in the termite cryptotermes secundus. *PloS one*, 14(2):e0210371.

Langfelder, P. and Horvath, S. (2008). Wgcna: an r package for weighted correlation network analysis. *BMC bioinformatics*, 9(1):559.

Laurindo, F. R., Pescatore, L. A., and de Castro Fernandes, D. (2012). Protein disulfide isomerase in redox cell signaling and homeostasis. *Free Radical Biology and Medicine*, 52(9):1954–1969.

Lee, B.-H., Lee, M. J., Park, S., Oh, D.-C., Elsasser, S., Chen, P.-C., Gartner, C., Dimova, N., Hanna, J., Gygi, S. P., et al. (2010). Enhancement of proteasome activity by a small-molecule inhibitor of usp14. *Nature*, 467(7312):179.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and Durbin, R. (2009). The sequence alignment/map format and samtools. *Bioinformatics*, 25(16):2078–2079.

Lin, Y.-J., Seroude, L., and Benzer, S. (1998). Extended life-span and stress resistance in the drosophila mutant methuselah. *Science*, 282(5390):943–946.

Loch, G., Zinke, I., Mori, T., Carrera, P., Schroer, J., Takeyama, H., and Hoch, M. (2017). Antimicrobial peptides extend lifespan in Drosophila. *PLoS ONE*, 12(5):1–15.

Lockett, G. A., Almond, E. J., Huggins, T. J., Parker, J. D., and Bourke, A. F. (2016). Gene expression differences in relation to age and social environment in queen and worker bumble bees. *Experimental gerontology*, 77:52–61.

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks of aging. *Cell*, 153(6):1194–1217.

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for rna-seq data with deseq2. *Genome biology*, 15(12):550.

Löytynoja, A. (2014). Phylogeny-aware alignment with prank. In *Multiple sequence alignment methods*, pages 155–170. Springer.

Lucas, E. R. and Keller, L. (2018). Elevated expression of ageing and immunity genes in queens of the black garden ant. *Experimental gerontology*, 108:92–98.

Lucas, E. R., Privman, E., and Keller, L. (2016). Higher expression of somatic repair genes in long-lived ant queens than workers. *Aging (Albany NY)*, 8(9):1940.

Lucas, E. R., Romiguier, J., and Keller, L. (2017). Gene expression is more strongly influenced by age than caste in the ant lasius niger. *Molecular ecology*, 26(19):5058–5073.

Mistry, J., Bateman, A., and Finn, R. D. (2007). Predicting active site residue annotations in the pfam database. *BMC bioinformatics*, 8(1):298.

Mitchell, A., Chang, H.-Y., Daugherty, L., Fraser, M., Hunter, S., Lopez, R., McAnulla, C., McMenamin, C., Nuka, G., Pesseat, S., et al. (2014). The interpro protein families database: the classification resource after 15 years. *Nucleic acids research*, 43(D1):D213–D221.

Negroni, M. A., Foitzik, S., and Feldmeyer, B. (2019a). Long-lived Temnothorax ant queens switch from investment in immunity to antioxidant production with age. *Scientific Reports*, 9(1):7270.

Negroni, M. A., Foitzik, S., and Feldmeyer, B. (2019b). Long-lived temnothorax ant queens switch from investment in immunity to antioxidant production with age. *Scientific reports*, 9(1):7270.

Negroni, M. A., Jongepier, E., Feldmeyer, B., Kramer, B. H., and Foitzik, S. (2016). Life history evolution in social insects: a female perspective. *Current opinion in insect science*, 16:51–57.

Oettler, J. and Schrempf, A. (2016). Fitness and aging in cardiocondyla obscurior ant queens. *Current opinion in insect science*, 16:58–63.

Partridge, L., Alic, N., Bjedov, I., and Piper, M. D. (2011). Ageing in drosophila: the role of the insulin/igf and tor signalling network. *Experimental gerontology*, 46(5):376–381.

R Core Team (2018). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Rivera, M. C., Jain, R., Moore, J. E., and Lake, J. A. (1998). Genomic evidence for two functionally distinct gene classes. *Proceedings of the National Academy of Sciences*, 95(11):6239–6244.

Rubinsztein, D. C., Mariño, G., and Kroemer, G. (2011). Autophagy and aging. *Cell*, 146(5):682–695.

Schrader, L., Kim, J. W., Ence, D., Zimin, A., Klein, A., Wyschetzki, K., Weichselgartner, T., Kemena, C., Stökl, J., Schultner, E., et al. (2014). Transposable element islands facilitate adaptation to novel environments in an invasive species. *Nature communications*, 5(1):1–10.

Schrempf, A., Giehr, J., Röhrl, R., Steigleder, S., and Heinze, J. (2017). Royal darwinian demons: enforced changes in reproductive efforts do not affect the life expectancy of ant queens. *The American Naturalist*, 189(4):436–442.

Schrempf, A., Heinze, J., and Cremer, S. (2005). Sexual cooperation: mating increases longevity in ant queens. *Current Biology*, 15(3):267–270.

Seehuus, S.-C., Taylor, S., Petersen, K., and Aamodt, R. M. (2013). Somatic maintenance resources in the honeybee worker fat body are distributed to withstand the most life-threatening challenges at each life stage. *PloS one*, 8(8):e69870.

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., Amin, N., Schwikowski, B., and Ideker, T. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome research*, 13(11):2498–2504.

Southworth, L. K., Owen, A. B., and Kim, S. K. (2009). Aging mice show a decreasing correlation of gene expression within genetic modules. *PLoS genetics*, 5(12):e1000776.

Strilbytska, O. M., Semaniuk, U. V., Storey, K. B., Edgar, B. A., and Lushchak, V. (2017). Activation of the tor/myc signaling axis in intestinal stem and progenitor cells affects longevity, stress resistance and metabolism in drosophila. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 203:92–99.

Suyama, M., Torrents, D., and Bork, P. (2006). Pal2nal: robust conversion of protein sequence alignments into the corresponding codon alignments. *Nucleic acids research*, 34(suppl_2):W609–W612.

Tatar, M., Bartke, A., and Antebi, A. (2003). The endocrine regulation of aging by insulin-like signals. *Science*, 299(5611):1346–1351.

Thurmond, J., Goodman, J. L., Strelets, V. B., Attrill, H., Gramates, L. S., Marygold, S. J., Matthews, B. B., Millburn, G., Antonazzo, G., Trovisco, V., et al. (2018). Flybase 2.0: the next generation. *Nucleic acids research*, 47(D1):D759–D765.

Tilstra, J. S., Robinson, A. R., Wang, J., Gregg, S. Q., Clauson, C. L., Reay, D. P., Nasto, L. A., St Croix, C. M., Usas, A., Vo, N., et al. (2012). Nf-κb inhibition delays dna damage–induced senescence and aging in mice. *The Journal of clinical investigation*, 122(7):2601–2612.

Tomaru, U., Takahashi, S., Ishizu, A., Miyatake, Y., Gohda, A., Suzuki, S., Ono, A., Ohara, J., Baba, T., Murata, S., et al. (2012). Decreased proteasomal activity causes age-related phenotypes and promotes the development of metabolic abnormalities. *The American journal of pathology*, 180(3):963–972.

Vilchez, D., Morantte, I., Liu, Z., Douglas, P. M., Merkwirth, C., Rodrigues, A. P., Manning, G., and Dillin, A. (2012). Rpn-6 determines c. elegans longevity under proteotoxic stress conditions. *Nature*, 489(7415):263.

Von Wyschetzki, K., Rueppell, O., Oettler, J., and Heinze, J. (2015). Transcriptomic signatures mirror the lack of the fecundity/longevity trade-off in ant queens. *Molecular biology and evolution*, 32(12):3173–3185.

Ward, P. S., Brady, S. G., Fisher, B. L., and Schultz, T. R. (2015). The evolution of myrmicine ants: phylogeny and biogeography of a hyperdiverse ant clade (h ymenoptera: F ormicidae). *Systematic Entomology*, 40(1):61–81.

Williams, G. C. (1957). Pleiotropy, natural selection, and the evolution of senescence. *evolution*, pages 398–411.

Yang, Z. (1997). Paml: a program package for phylogenetic analysis by maximum likelihood. *Bioinformatics*, 13(5):555–556.

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Contributions

MCH, EBB conceived and initiated the project. MCH, EBB and JO designed the study. MCH wrote the manuscript and carried out most analyses. JR assisted with dN/dS analyses. MCH and JO interpreted ant data, all authors interpreted comparative data. LMJ assisted in the interpretation of GO term enrichment analyses. TF and MAR generated fly data used in IIS/TOR analysis and helped analyse them. MCH wrote the manuscript which was revised and approved by all authors.

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Data Availability

Ant queen data are already published (Von Wyschetzki et al., 2015) and available at SRA under accessions: PRJNA293450 & PRJNA284224. *Drosophila* data are deposited on SRA under accession: PRJNA615318. All other information is available on request.

675 ## Supplementary figures

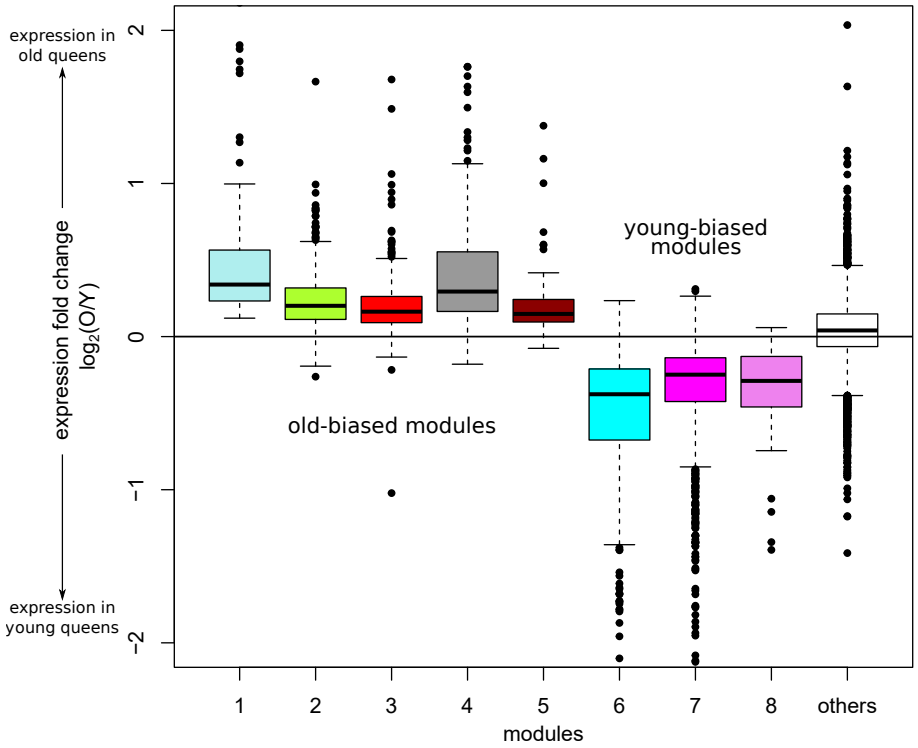

Figure S1: Fold change of expression in old compared to young queens in each of the significant modules ($\log_2$(old/young)). Correspondingly, genes within the 'old-biased' modules (1-5) show $\log_2$-fold-change of expression > 0 (medians: 0.340, 0.201, 0.163, 0.294, 0.148, respectively) and 'young-biased' modules (6-8) contain genes with negative $\log_2$-fold-change of expression (medians: -0.376, -0.249, -0.289, respectively). Expression fold change for genes of all other modules (white plot, right-most), on the other hand, has a median close to zero (0.040).

676 ## Supplementary Tables

Table S1: GO terms (Biological Process) significantly enriched within genes with a connectivity fold change greater than 2 in old compared to young queens.

GO.ID	Term	Annotated	Significant	Expected	pvalue	FDR
GO:0006518	peptide metabolic process	168	67	20.85	1.1e-20	3.4e-18
GO:0043043	peptide biosynthetic process	163	65	20.23	4.5e-20	6.4e-18
GO:0006412	translation	160	64	19.85	7.5e-20	7.7e-18
GO:0043604	amide biosynthetic process	166	65	20.6	1.4e-19	9.8e-18
GO:0043603	cellular amide metabolic process	175	67	21.71	1.6e-19	9.8e-18
GO:1901566	organonitrogen compound biosynthetic pro...	270	83	33.5	3.8e-17	1.9e-15
GO:0044267	cellular protein metabolic process	574	128	71.22	1e-13	4.4e-12

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Table S1 – *Continued from previous page*

GO.ID	Term	Annotated	Significant	Expected	pvalue	FDR
GO:0019538	protein metabolic process	744	141	92.32	2.5e-09	9.6e-08
GO:1901564	organonitrogen compound metabolic proces...	892	161	110.68	4.5e-09	1.5e-07
GO:0034645	cellular macromolecule biosynthetic proc...	544	106	67.5	1.3e-07	4.0e-06
GO:0009059	macromolecule biosynthetic process	546	106	67.75	1.6e-07	4.3e-06
GO:0044271	cellular nitrogen compound biosynthetic ...	553	107	68.62	1.7e-07	4.3e-06
GO:0009058	biosynthetic process	700	128	86.86	2.2e-07	5.2e-06
GO:0044249	cellular biosynthetic process	655	121	81.28	3.1e-07	6.8e-06
GO:1901576	organic substance biosynthetic process	664	122	82.39	3.7e-07	7.6e-06
GO:0009987	cellular process	1960	288	243.21	5.8e-07	1.1e-5
GO:0044260	cellular macromolecule metabolic process	1029	170	127.68	1.4e-06	2.5e-5
GO:0044237	cellular metabolic process	1425	220	176.82	2.8e-06	4.8e-5
GO:0010467	gene expression	598	107	74.2	1e-05	1.6e-4
GO:0034641	cellular nitrogen compound metabolic pro...	872	141	108.2	7.6e-5	0.001
GO:0006807	nitrogen compound metabolic process	1494	216	185.38	7.0e-4	0.010
GO:0008152	metabolic process	2076	286	257.6	9.6e-4	0.013
GO:0044238	primary metabolic process	1584	226	196.55	0.001	0.014
GO:0045333	cellular respiration	8	5	0.99	0.001	0.015
GO:0043170	macromolecule metabolic process	1370	198	170	0.002	0.020
GO:0046034	ATP metabolic process	25	9	3.1	0.002	0.025
GO:0071704	organic substance metabolic process	1665	234	206.6	0.002	0.025
GO:0015980	energy derivation by oxidation of organi...	9	5	1.12	0.002	0.026
GO:0009144	purine nucleoside triphosphate metabolic...	26	9	3.23	0.003	0.028
GO:0009199	ribonucleoside triphosphate metabolic pr...	26	9	3.23	0.003	0.028
GO:0009205	purine ribonucleoside triphosphate metab...	26	9	3.23	0.003	0.028
GO:0009123	nucleoside monophosphate metabolic proce...	27	9	3.35	0.004	0.033
GO:0009126	purine nucleoside monophosphate metaboli...	27	9	3.35	0.004	0.033
GO:0009141	nucleoside triphosphate metabolic proces...	27	9	3.35	0.004	0.033
GO:0009161	ribonucleoside monophosphate metabolic p...	27	9	3.35	0.004	0.033
GO:0009167	purine ribonucleoside monophosphate meta...	27	9	3.35	0.004	0.033
GO:0022900	electron transport chain	7	4	0.87	0.006	0.050
GO:0006091	generation of precursor metabolites and ...	20	7	2.48	0.008	0.063
GO:0019693	ribose phosphate metabolic process	44	11	5.46	0.016	0.122
GO:0007049	cell cycle	33	9	4.09	0.016	0.122
GO:0009056	catabolic process	82	17	10.18	0.021	0.149
GO:0006754	ATP biosynthetic process	19	6	2.36	0.023	0.149
GO:0009142	nucleoside triphosphate biosynthetic pro...	19	6	2.36	0.023	0.149
GO:0009145	purine nucleoside triphosphate biosynthe...	19	6	2.36	0.023	0.149
GO:0009201	ribonucleoside triphosphate biosynthetic...	19	6	2.36	0.023	0.149
GO:0009206	purine ribonucleoside triphosphate biosy...	19	6	2.36	0.023	0.149
GO:0044257	cellular protein catabolic process	35	9	4.34	0.023	0.149
GO:0051603	proteolysis involved in cellular protein...	35	9	4.34	0.023	0.149
GO:0019637	organophosphate metabolic process	116	22	14.39	0.025	0.159
GO:0007005	mitochondrion organization	10	4	1.24	0.027	0.163
GO:0044248	cellular catabolic process	72	15	8.93	0.028	0.165
GO:0006888	ER to Golgi vesicle-mediated transport	6	3	0.74	0.028	0.165
GO:0009150	purine ribonucleotide metabolic process	42	10	5.21	0.029	0.165
GO:0009259	ribonucleotide metabolic process	42	10	5.21	0.029	0.165

Continued on next page

Table S1 – *Continued from previous page*

GO.ID	Term	Annotated	Significant	Expected	pvalue	FDR
GO:0009124	nucleoside monophosphate biosynthetic pr...	21	6	2.61	0.037	0.193
GO:0009127	purine nucleoside monophosphate biosynth...	21	6	2.61	0.037	0.193
GO:0009156	ribonucleoside monophosphate biosyntheti...	21	6	2.61	0.037	0.193
GO:0009168	purine ribonucleoside monophosphate bios...	21	6	2.61	0.037	0.193
GO:0015985	energy coupled proton transport, down el...	11	4	1.36	0.038	0.193
GO:0015986	ATP synthesis coupled proton transport	11	4	1.36	0.038	0.193
GO:0030163	protein catabolic process	38	9	4.72	0.039	0.194
GO:1901137	carbohydrate derivative biosynthetic pro...	76	15	9.43	0.043	0.210
GO:1901575	organic substance catabolic process	76	15	9.43	0.043	0.210
GO:0044265	cellular macromolecule catabolic process	45	10	5.58	0.045	0.210
GO:0006839	mitochondrial transport	7	3	0.87	0.045	0.210
GO:1990542	mitochondrial transmembrane transport	7	3	0.87	0.045	0.210
GO:1901135	carbohydrate derivative metabolic proces...	144	25	17.87	0.048	0.218

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Table S2: Genes with the greatest increase in connectivity in old compared to young queens.

	C. obscurior gene	Connectivity fold change (old/young)	Ortholog in *D. melanogaster*	E-value	PFAM domains	Function	GCN Module
1	Cobs_00057	7.1	FBpp0079031 (Suppressor of exocyst mutations 1)	2e-12	PF05160.12:DSS1_SEM1	member of 26S proteasome complex	27
2	Cobs_01666	6.5	FBpp0297101 (Fatty acid synthase 3)	0.0	PF00698.20:Acyl_transf_1 PF00975.19:Thioesterase PF14765.5:PS-DH PF16197.4:KAsynt_C_assoc PF08659.9:KR PF00109.25:ketoacyl-synt PF00107.25:ADH_zinc_N PF00550.24:PP-binding PF02801.21:Ketoacyl-synt_C	fatty acid synthesis	3
3	Cobs_03333	6.3	Golgi apparatus membrane protein TVP23 homolog B (Q9NYZ1)	0.014	/	unknown	16
4	Cobs_09457	6.1	FBpp0081645 (CG12948)	1e-21	PF14969.5:DUF4508	unknown	14
5	Cobs_03249	5.9	FBpp0083650 (Prefoldin 5)	0.001	/	protein folding	3
6	Cobs_18104	5.7	Motile sperm domain-containing protein 2 (Q8NHP6)	4e-66	PF00650.19:CRAL_TRIO PF00635.25:Motile_Sperm	ER protein, promotes interorganelle contacts	15
7	Cobs_08529	5.6	phospholipase A1 (Q68KK0)	4e-93	PF00151:Lipase	phospholipase	18
8	Cobs_06641	5.6	FBpp0079845 (FBpp0079845)	5e-46	PF00096.25:zf-C2H2 PF12874.6:zf-met PF13912.5:zf-C2H2_6	transcription factor	10
9	Cobs_07556	5.5	FBpp0074522 (CG14229)	8e-14	/	unknown	27
10	Cobs_07129	5.4	FBpp0070766 (RpL35-PB)	7e-63	PF00831:Ribosomal_L29	ribosomal protein	27
11	Cobs_15323	5.4	FBpp0086393 (Polynucleotide 5'-hydroxyl-kinase)	2e-31	PF16575.4:CLP1_P	mRNA cleavage and polyadenylation factor, Clp1	10
12	Cobs_12967	5.4	FBpp0308983 (combgap)	1e-154	PF00096.25:zf-C2H2	transcription factor	2
13	Cobs_10359	5.4	FBpp0083078 (CG31229)	4e-87	PF02466:Tim17	Mitochondrial import inner membrane translocase subunit Tim22	3
14	Cobs_09306	5.3	FBpp0074936 (RNA helicase)	0.0	PF00270:DEAD, PF00271:Helicase_C	RNA helicase	10
15	Cobs_09326	5.2	FBpp0079752 (RpL9)	7e-109	PF00347:Ribosomal_L6	ribosomal protein	27
16	Cobs_17451	5.2	FBpp0081234 (SmD2)	3e-67	PF01423:LSM	Small ribonucleoprotein particle protein; splicing	16
17	Cobs_01606	5.0	FBpp0290896 (CG31690)	1e-117	PF08409:DUF1736	Protein O-mannosyl-transferase (protein modification)	27
18	Cobs_14874	5.0	FBpp0084171 (Smg6)	3e-40	PF10373:EST1_DNA_bind, PF13638:PIN_4	nonsense mediated mRNA decay	10
19	Cobs_03737	4.9	FBpp0073777 (ND-B18)	4e-38	PF05676:NDUF_B7	NADH dehydrogenase (ubiquinone) B18 subunit	27
20	Cobs_09935	4.1	FBpp0082711 (taranis)	2e-21	PF06031.12:SERTA	transcriptional co-regulator, chromatin remodelling	20

Table S3: Top hubs of discussed modules within the GCN.

	C. obscurior gene	*D. melanogaster* ortholog	pfam domains	putative function
module_1				
1	Cobs_16506	Fatty acid synthase (Q71SP7)	Acyl_transf_1	fatty-acid synthase
2	Cobs_15810	FBgn0013726	Septin	septin
3	Cobs_13037	FBgn0037022	NA	TRAPP complex, protein transport
4	Cobs_02638	Glutaredoxin domain-containing cysteine-rich protein (Q9VNL4)	NA	GRXCR1, post-transcriptional S-glutathionylation
5	Cobs_16282	FBgn0037238	Na_Ca_ex	Ca(2+):cation antiporter
6	Cobs_13547	FBgn0033266	SH2,SOCS_box	Socs44A, ubiquitination
7	Cobs_03654	FBgn0001104	G-alpha	G protein α i subunit
8	Cobs_00923	NA	NA	
9	Cobs_06675	FBgn0052702	EGF,hEGF, EGF_CA,EGF_3,CUB	
10	Cobs_06885	FBgn0261556	RhoGEF	guanine nucleotide exchange factor
module_2				
1	Cobs_13808	Helicase MOV-10 (Q9HCE1)	AAA_11	RNA helicase (mov-10-B.1)
2	Cobs_05444	NA	NA	
3	Cobs_07118	FBgn0000615	NA	exuperantia - maternal protein, polarity of the oocyte
4	Cobs_12340	FBgn0035914	DUF1295	oxidoreductase, uncharacterised
5	Cobs_11183	Furin (P23188)	4e-4	furin-like protease 2 activation of precursor proteins
6	Cobs_00943	NA	NA	fasciclin-2: Neuronal recognition molecule
7	Cobs_05806	FBgn0066365	Zona_pellucida	dusky-like, cuticle
8	Cobs_04366	Venom dipeptidyl peptidase 4 (B2D0J4)	DPPIV_N	venom dipeptidyl peptidase 4
9	Cobs_10253	FBgn0051719	PseudoU_synth_2	RluA pseudouridine synthase 1
10	Cobs_11743	FBgn0040342	PPDK_N, PEP-utilizers	putative phosphoenolpyruvate synthase
module_3				
1	Cobs_10830	Serine/threonine/tyrosine-interacting protein (Q60969)	DSPc	STYX: ubiquitination & MAPK signalling
2	Cobs_11033	FBgn0035590	Kdo	KEOPS/EKC: transcr. regulation
3	Cobs_08034	FBgn0031403	P_C10	
4	Cobs_11836	FBgn0033663	Thioredoxin, Thioredoxin_6	ER stress protein, disulfide-isomerase
5	Cobs_16500	Fringe glycosyltransferase (Q24342)	Fringe	fringe, Notch signalling
6	Cobs_03447	NA	NA	
7	Cobs_02166	FBgn0033644	Sugar_tr	trehalose transporter
8	Cobs_01536	FBgn0030434	Sds3	histone deacetylase
9	Cobs_08376	FBgn0039233	UPF0113	Nip, ribosome assembly
10	Cobs_16030	FBgn0030871	AAA, Rep_fac_C	part of DNA clamp
module_4				
1	Cobs_01588	FBgn0082582	Tropomodulin	actin filaments in muscles
2	Cobs_13880	FBgn0010497	MFS_1	Dietary and metabolic glutamate transporter
3	Cobs_13613	FBgn0034647	NA	poor Imd response upon knock-in (pink) negative regulator of Imd pathway
4	Cobs_10261	FBgn0032235	LRR_8, LRR_1	Leucine-rich repeat transmembrane neuronal protein 2
5	Cobs_09494	FBgn0023507	FAD_binding_4,FAD-oxidase_C	D-2-hydroxyglutaric acid dehydrogenase mitochonrion metabolism
6	Cobs_18099	Krueppel-like factor luna (Q8MR37)	NA	Krueppel-like factor 7 TF,nucleic acid binding
7	Cobs_18085	Protein G12 (Q17040)	Ins_allergen_rp	Protein G12 Heterotrimeric G protein cytoskeleton
8	Cobs_16115	Chymotrypsin-1 (Q7SIG2)	Trypsin	Chymotrypsin-1 digestive enzyme

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Table S3 – *Continued from previous page*

	C. obscurior gene	*D. melanogaster* ortholog	pfam domains	putative function
9	Cobs_12564	FBgn0032381	Alpha-amylase	alpha glucosidase digestive
10	Cobs_11149	Odorant receptor 13a	7tm_6	odorant receptor
module_5				
1	Cobs_14833	FBgn0261434	THAP,zf-C2H2	Huckebein, DNA binding
2	Cobs_03225	NA	NA	
3	Cobs_03424	NA	NA	
4	Cobs_05905	FBgn0036460	WD40	AAMP - angiogenesis
5	Cobs_09948	FBgn0000320	NA	protein-serine/threonine phosphate
6	Cobs_10152	Fatty acid synthase (P19096)	ketoacyl-synt, Ketoacyl-synt_C, KAsynt_C_assoc,Acyl_transf_1, PS-DH,ADH_zinc_N,KR,PP-binding, Thioesterase	fatty-acid synthesis
7	Cobs_11210	COMM domain-containing protein 8 (Q9CZG3)	COMM_domain	
8	Cobs_08984	FBgn0033507	zf-LYAR	DNA binding
9	Cobs_02509	FBgn0039623	Pkinase	intracellular trafficking
10	Cobs_10282	FBgn0030878	zf-met,zf-C2H2_2	TF
module_6				
1	Cobs_05316	Thrombospondin type-1 domain-containing protein 4 (Q3UTY6)	TSP_1, ADAM_spacer1	thrombospondin type 1
2	Cobs_09783	FBgn0034578	Coa1	Cytochrome oxidase complex assembly
3	Cobs_16775	NA	NA	
4	Cobs_10977	NA	NA	
5	Cobs_05884	FBgn0052264	RPEL	actin binding
6	Cobs_08339	Putative odorant receptor 71a (Q9VUK5)	7tm_6	odorant receptor
7	Cobs_07520	FBgn0030174	I-set, fn3	NA
8	Cobs_06555	Protein BTG3 (P50615)	BTG	negative regulator of cell cycle
9	Cobs_06011	NA	NA	NA
10	Cobs_16771	FBgn0004169	Troponin	muscle protein
module_7				
1	Cobs_10334	NA	NA	
2	Cobs_07055	NA	NA	
3	Cobs_09646	FBgn0024986	Thioredoxin	protein disulfide oxidoreductase activity
4	Cobs_11762	FBgn0250843	Proteasome_A_N	Proteasome
5	Cobs_04738	FBgn0027052	TPR_16, TPR_8, U-box	STUB1, insulin signalling & ubiquitination
6	Cobs_17742	FBgn0002937	RPE65	rhodopsin/vitamin biosynthesis
7	Cobs_07132	FBgn0004436	UQ_con	Ubiquitin conjugating enzyme 6
8	Cobs_16235	NA	NA	
9	Cobs_16742	FBgn0051005	polyprenyl_synt	qless, CoenzymeQ synthesis
10	Cobs_08806	FBgn0036133	Tmemb_161AB	
module_8				
1	Cobs_08138	FBgn0035132	7tm_2	methuselah (mth) modulation of life span & stress response
2	Cobs_06914	FBgn0015808	Thiolase_N, Thiolase_C, SCP2	phospholipid transporter activity
3	Cobs_07075	FBgn0058470	Peptidase_M1, ERAP1_C	
4	Cobs_08585	NA	NA	
5	Cobs_04843	FBgn0037637	NifU_N	Iron-sulfur cluster assembly enzyme
6	Cobs_00768	High-affinity choline transporter 1 (Q9VE46)	SSF	
7	Cobs_15292	NA	NA	
8	Cobs_07928	FBgn0052626	A_deaminase	AMP deaminase
9	Cobs_03203	FBgn0243512	DSPc	serine/threonine protein phosphatase regulates Jun-N-terminal kinase pathway
. 10	Cobs_07588	AMMECR1-like protein (Q8JZZ6)	AMMECR1	
module_27				

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Table S3 – *Continued from previous page*

	C. obscurior gene	*D. melanogaster* ortholog	pfam domains	putative function
1	Cobs_17974	FBgn0035998	SAC3_GANP	SAC3 domain-containing protein 1 centrosome duplication & mitotic progression
2	Cobs_16539	NA	NA	NA
3	Cobs_08044	FBgn0000618	ENY2	enhancer of yellow 2 nuclear export of mRNA transcription activation
4	Cobs_06960	FBgn0036545	Glutaredoxin, PLA2G12	GXIVsPLA2 activation of IMD pathway
5	Cobs_17771	18S rRNA aminocarboxypropyl-transferase (Q5HZH2)	RLI, Ribo_biogen_C	Ribosome biogenesis protein TSR3 homolog
6	Cobs_04280	FBgn0051251	CS, Nudc_N	dynein stability
7	Cobs_17249	FBgn0024983	ERGIC_N, COPIIcoated_ERV	transport between ER & Golgi
8	Cobs_15425	FBgn0261597	Ribosomal_S26e	Ribosomal protein S26
9	Cobs_09453	NA	NA	BAI1-associated protein endosome to Golgi transport
10	Cobs_08415	NA	SEFIR	possible TOLL/IL1R-like signalling

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Table S4: GO terms (Biological Process) significantly enriched within selected modules of the GCN.

GO.ID	Term	Annotated	Significant	Expected	pvalue	FDR
module_1						
GO:0006357	regulation of transcription by RNA polym...	22	3	0.2	0.001	0.075
GO:0016192	vesicle-mediated transport	68	4	0.62	0.003	0.115
GO:0006366	transcription by RNA polymerase II	37	3	0.34	0.004	0.115
GO:0006886	intracellular protein transport 70	3	0.64	0.025	0.231	
GO:0034613	cellular protein localization	77	3	0.7	0.032	0.231
GO:0070727	cellular macromolecule localization	77	3	0.7	0.032	0.231
GO:0015031	protein transport	82	3	0.75	0.038	0.231
GO:0015833	peptide transport	82	3	0.75	0.038	0.231
GO:0046907	intracellular transport	82	3	0.75	0.038	0.231
GO:0051649	establishment of localization in cell	82	3	0.75	0.038	0.231
GO:0042886	amide transport	83	3	0.76	0.039	0.231
GO:0045184	establishment of protein localization	83	3	0.76	0.039	0.231
GO:0008104	protein localization	89	3	0.81	0.046	0.231
module_2						
GO:0006464	cellular protein modification process	373	24	14.44	0.008	0.277
GO:0036211	protein modification process	373	24	14.44	0.008	0.277
GO:0007018	microtubule-based movement	46	6	1.78	0.008	0.277
GO:0043412	macromolecule modification	397	25	15.37	0.008	0.277
GO:0006928	movement of cell or subcellular componen...	48	6	1.86	0.010	0.277
GO:0016579	protein deubiquitination	28	4	1.08	0.021	0.361
GO:0070646	protein modification by small protein re...	28	4	1.08	0.021	0.361
GO:0070647	protein modification by small protein co...	43	5	1.66	0.024	0.361
GO:0007017	microtubule-based process	61	6	2.36	0.029	0.361
GO:0044260	cellular macromolecule metabolic process	1029	49	39.84	0.047	0.361
GO:0006355	regulation of transcription, DNA-templat...	245	15	9.49	0.049	0.361
GO:0051252	regulation of RNA metabolic process	245	15	9.49	0.049	0.361
GO:1903506	regulation of nucleic acid-templated tra...	245	15	9.49	0.049	0.361
GO:2001141	regulation of RNA biosynthetic process	245	15	9.49	0.049	0.361
module_3						
GO:0016051	carbohydrate biosynthetic process	9	3	0.33	0.003	0.276
GO:0033365	protein localization to organelle	20	4	0.73	0.005	0.276
GO:0065008	regulation of biological quality	60	7	2.2	0.006	0.276
GO:0051641	cellular localization	101	9	3.7	0.011	0.388
GO:0034613	cellular protein localization	77	7	2.82	0.021	0.464
GO:0070727	cellular macromolecule localization	77	7	2.82	0.021	0.464
GO:0045454	cell redox homeostasis	30	4	1.1	0.022	0.464
GO:0008610	lipid biosynthetic process	32	4	1.17	0.028	0.464

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Table S4 – *Continued from previous page*

GO.ID	Term	Annotated	Significant	Expected	pvalue	FDR
GO:0019725	cellular homeostasis	35	4	1.28	0.037	0.464
GO:0008104	protein localization	89	7	3.26	0.043	0.464
GO:0006357	regulation of transcription by RNA polym...	22	3	0.8	0.044	0.464
GO:0006950	response to stress	73	6	2.67	0.049	0.464
GO:0046903	secretion	23	3	0.84	0.050	0.464
module_4						
GO:0055085	transmembrane transport	283	13	5	0.001	0.066
GO:0006508	proteolysis	233	10	4.12	0.007	0.172
GO:0006810	transport	544	17	9.62	0.011	0.172
GO:0051234	establishment of localization	545	17	9.64	0.011	0.172
GO:0051179	localization	556	17	9.83	0.013	0.172
GO:0006812	cation transport	92	5	1.63	0.022	0.243
GO:0048519	negative regulation of biological proces...	38	3	0.67	0.029	0.259
GO:0006030	chitin metabolic process	43	3	0.76	0.039	0.259
GO:0006040	amino sugar metabolic process	43	3	0.76	0.039	0.259
GO:1901071	glucosamine-containing compound metaboli...	43	3	0.76	0.039	0.259
module_5						
GO:0071705	nitrogen compound transport	100	5	1.25	0.008	0.205
GO:0019222	regulation of metabolic process	270	8	3.38	0.017	0.205
GO:0015031	protein transport	82	4	1.03	0.018	0.205
GO:0015833	peptide transport	82	4	1.03	0.018	0.205
GO:0034660	ncRNA metabolic process	82	4	1.03	0.018	0.205
GO:0042886	amide transport	83	4	1.04	0.019	0.205
GO:0045184	establishment of protein localization	83	4	1.04	0.019	0.205
GO:0071702	organic substance transport	126	5	1.58	0.019	0.205
GO:0008104	protein localization	89	4	1.11	0.024	0.210
GO:0044085	cellular component biogenesis	92	4	1.15	0.027	0.210
GO:0071840	cellular component organization or bioge...	190	6	2.38	0.029	0.210
GO:0065003	protein-containing complex assembly	56	3	0.7	0.032	0.210
GO:0043933	protein-containing complex subunit organ...	61	3	0.76	0.040	0.210
GO:0031323	regulation of cellular metabolic process	261	7	3.26	0.040	0.210
GO:0051171	regulation of nitrogen compound metaboli...	261	7	3.26	0.040	0.210
GO:0080090	regulation of primary metabolic process	261	7	3.26	0.040	0.210
GO:0022607	cellular component assembly	64	3	0.8	0.045	0.210
GO:0060255	regulation of macromolecule metabolic pr...	269	7	3.36	0.046	0.210
GO:0006399	tRNA metabolic process	65	3	0.81	0.046	0.210
GO:0016043	cellular component organization	162	5	2.02	0.049	0.211
module_6						
GO:0055085	transmembrane transport	283	40	22.09	9.7e-05	0.015
GO:0006813	potassium ion transport	10	5	0.78	5.1e-4	0.039
GO:0006810	transport	544	57	42.46	0.009	0.202
GO:0051234	establishment of localization	545	57	42.54	0.009	0.202
GO:0007154	cell communication	334	38	26.07	0.009	0.202
GO:0023052	signaling	334	38	26.07	0.009	0.202
GO:0009165	nucleotide biosynthetic process	58	10	4.53	0.013	0.202
GO:1901293	nucleoside phosphate biosynthetic proces...	58	10	4.53	0.013	0.202
GO:0051179	localization	556	57	43.4	0.013	0.202
GO:0006811	ion transport	194	24	15.14	0.014	0.202
GO:0051259	protein complex oligomerization	13	4	1.01	0.015	0.202
GO:0007186	G protein-coupled receptor signaling pat...	126	17	9.83	0.017	0.209
GO:0007165	signal transduction	327	36	25.52	0.018	0.209
GO:0006814	sodium ion transport	14	4	1.09	0.019	0.209
GO:0009108	coenzyme biosynthetic process	22	5	1.72	0.024	0.245
GO:0030001	metal ion transport	47	8	3.67	0.027	0.245
GO:0009187	cyclic nucleotide metabolic process	23	5	1.8	0.029	0.245
GO:0009190	cyclic nucleotide biosynthetic process	23	5	1.8	0.029	0.245
GO:0009117	nucleotide metabolic process	71	10	5.54	0.047	0.354
GO:0051260	protein homooligomerization	11	3	0.86	0.048	0.354
module_7						
GO:0055114	oxidation-reduction process	268	84	37.67	1.9e-14	6.3e-12
GO:0044281	small molecule metabolic process	178	51	25.02	1.4e-07	2.3e-05
GO:0005975	carbohydrate metabolic process	96	32	13.49	9.2e-07	1.0e-4
GO:0008152	metabolic process	2076	327	291.78	1.2e-4	0.010
GO:0019693	ribose phosphate metabolic process	44	16	6.18	1.7e-4	0.010

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Table S4 – *Continued from previous page*

GO.ID	Term	Annotated	Significant	Expected	pvalue	FDR
GO:0019637	organophosphate metabolic process	116	31	16.3	1.9e-4	0.010
GO:0006163	purine nucleotide metabolic process	46	16	6.47	3.0e-4	0.012
GO:0009150	purine ribonucleotide metabolic process	42	15	5.9	3.4e-4	0.012
GO:0009259	ribonucleotide metabolic process	42	15	5.9	3.4e-4	0.012
GO:0072521	purine-containing compound metabolic pro...	47	16	6.61	4.0e-4	0.013
GO:0055086	nucleobase-containing small molecule met...	82	23	11.53	6.1e-4	0.018
GO:0019752	carboxylic acid metabolic process	79	22	11.1	8.8e-4	0.019
GO:0006164	purine nucleotide biosynthetic process	37	13	5.2	0.001	0.019
GO:0006629	lipid metabolic process	95	25	13.35	0.001	0.019
GO:0006082	organic acid metabolic process	80	22	11.24	0.001	0.019
GO:0043436	oxoacid metabolic process	80	22	11.24	0.001	0.019
GO:0009152	purine ribonucleotide biosynthetic proce...	33	12	4.64	0.001	0.019
GO:0009260	ribonucleotide biosynthetic process	33	12	4.64	0.001	0.019
GO:0046390	ribose phosphate biosynthetic process	33	12	4.64	0.001	0.019
GO:0051186	cofactor metabolic process	42	14	5.9	0.001	0.019
GO:0072522	purine-containing compound biosynthetic ...	38	13	5.34	0.001	0.021
GO:0044282	small molecule catabolic process	8	5	1.12	0.002	0.031
GO:0006732	coenzyme metabolic process	27	10	3.79	0.002	0.034
GO:0019439	aromatic compound catabolic process	23	9	3.23	0.003	0.034
GO:1901361	organic cyclic compound catabolic proces...	23	9	3.23	0.003	0.034
GO:0009117	nucleotide metabolic process	71	19	9.98	0.003	0.041
GO:0055085	transmembrane transport	283	56	39.78	0.003	0.041
GO:0032787	monocarboxylic acid metabolic process	16	7	2.25	0.004	0.044
GO:0006753	nucleoside phosphate metabolic process	72	19	10.12	0.004	0.044
GO:0016052	carbohydrate catabolic process	9	5	1.26	0.004	0.046
GO:0046034	ATP metabolic process	25	9	3.51	0.005	0.053
GO:0044255	cellular lipid metabolic process	54	15	7.59	0.006	0.054
GO:0044283	small molecule biosynthetic process	30	10	4.22	0.006	0.054
GO:0009144	purine nucleoside triphosphate metabolic...	26	9	3.65	0.007	0.054
GO:0009199	ribonucleoside triphosphate metabolic pr...	26	9	3.65	0.007	0.054
GO:0009205	purine ribonucleoside triphosphate metab...	26	9	3.65	0.007	0.054
GO:0009166	nucleotide catabolic process	10	5	1.41	0.007	0.054
GO:0009108	coenzyme biosynthetic process	22	8	3.09	0.008	0.054
GO:0044270	cellular nitrogen compound catabolic pro...	22	8	3.09	0.008	0.054
GO:0046700	heterocycle catabolic process	22	8	3.09	0.008	0.054
GO:0009056	catabolic process	82	20	11.53	0.008	0.054
GO:0006733	oxidoreduction coenzyme metabolic proces...	14	6	1.97	0.008	0.054
GO:0044248	cellular catabolic process	72	18	10.12	0.009	0.054
GO:0009123	nucleoside monophosphate metabolic proce...	27	9	3.79	0.009	0.054
GO:0009126	purine nucleoside monophosphate metaboli...	27	9	3.79	0.009	0.054
GO:0009141	nucleoside triphosphate metabolic proces...	27	9	3.79	0.009	0.054
GO:0009161	ribonucleoside monophosphate metabolic p...	27	9	3.79	0.009	0.054
GO:0009167	purine ribonucleoside monophosphate meta...	27	9	3.79	0.009	0.054
GO:0015908	fatty acid transport	7	4	0.98	0.009	0.054
GO:0015909	long-chain fatty acid transport	7	4	0.98	0.009	0.054
GO:0016054	organic acid catabolic process	7	4	0.98	0.009	0.054
GO:0032309	icosanoid secretion	7	4	0.98	0.009	0.054
GO:0046395	carboxylic acid catabolic process	7	4	0.98	0.009	0.054
GO:0046717	acid secretion	7	4	0.98	0.009	0.054
GO:0050482	arachidonic acid secretion	7	4	0.98	0.009	0.054
GO:0071715	icosanoid transport	7	4	0.98	0.009	0.054
GO:1901571	fatty acid derivative transport	7	4	0.98	0.009	0.054
GO:1903963	arachidonate transport	7	4	0.98	0.009	0.054
GO:0006820	anion transport	32	10	4.5	0.010	0.054
GO:0006754	ATP biosynthetic process	19	7	2.67	0.011	0.056
GO:0009142	nucleoside triphosphate biosynthetic pro...	19	7	2.67	0.011	0.056
GO:0009145	purine nucleoside triphosphate biosynthe...	19	7	2.67	0.011	0.056
GO:0009201	ribonucleoside triphosphate biosynthetic...	19	7	2.67	0.011	0.056
GO:0009206	purine ribonucleoside triphosphate biosy...	19	7	2.67	0.011	0.056
GO:0005996	monosaccharide metabolic process	11	5	1.55	0.012	0.056
GO:0015718	monocarboxylic acid transport	11	5	1.55	0.012	0.056
GO:0015849	organic acid transport	11	5	1.55	0.012	0.056
GO:0019318	hexose metabolic process	11	5	1.55	0.012	0.056
GO:0046942	carboxylic acid transport	11	5	1.55	0.012	0.056

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GO.ID	Term	Annotated	Significant	Expected	pvalue	FDR
GO:1901292	nucleoside phosphate catabolic process	11	5	1.55	0.012	0.056
GO:1901605	alpha-amino acid metabolic process	11	5	1.55	0.012	0.056
GO:1901575	organic substance catabolic process	76	18	10.68	0.016	0.064
GO:0006090	pyruvate metabolic process	8	4	1.12	0.017	0.064
GO:0006096	glycolytic process	8	4	1.12	0.017	0.064
GO:0006165	nucleoside diphosphate phosphorylation	8	4	1.12	0.017	0.064
GO:0006757	ATP generation from ADP	8	4	1.12	0.017	0.064
GO:0009132	nucleoside diphosphate metabolic process	8	4	1.12	0.017	0.064
GO:0009135	purine nucleoside diphosphate metabolic ...	8	4	1.12	0.017	0.064
GO:0009179	purine ribonucleoside diphosphate metabo...	8	4	1.12	0.017	0.064
GO:0009185	ribonucleoside diphosphate metabolic pro...	8	4	1.12	0.017	0.064
GO:0030258	lipid modification	8	4	1.12	0.017	0.064
GO:0042866	pyruvate biosynthetic process	8	4	1.12	0.017	0.064
GO:0046031	ADP metabolic process	8	4	1.12	0.017	0.064
GO:0046939	nucleotide phosphorylation	8	4	1.12	0.017	0.064
GO:0016053	organic acid biosynthetic process	16	6	2.25	0.017	0.064
GO:0046394	carboxylic acid biosynthetic process	16	6	2.25	0.017	0.064
GO:1902600	proton transmembrane transport	25	8	3.51	0.017	0.064
GO:0019362	pyridine nucleotide metabolic process	12	5	1.69	0.018	0.064
GO:0034404	nucleobase-containing small molecule bio...	12	5	1.69	0.018	0.064
GO:0046434	organophosphate catabolic process	12	5	1.69	0.018	0.064
GO:0046496	nicotinamide nucleotide metabolic proces...	12	5	1.69	0.018	0.064
GO:0072330	monocarboxylic acid biosynthetic process	12	5	1.69	0.018	0.064
GO:0072524	pyridine-containing compound metabolic p...	12	5	1.69	0.018	0.064
GO:0051188	cofactor biosynthetic process	30	9	4.22	0.018	0.064
GO:0009124	nucleoside monophosphate biosynthetic pr...	21	7	2.95	0.020	0.068
GO:0009127	purine nucleoside monophosphate biosynth...	21	7	2.95	0.020	0.068
GO:0009156	ribonucleoside monophosphate biosyntheti...	21	7	2.95	0.020	0.068
GO:0009168	purine ribonucleoside monophosphate bios...	21	7	2.95	0.020	0.068
GO:0009063	cellular amino acid catabolic process	5	3	0.7	0.022	0.072
GO:0046834	lipid phosphorylation	5	3	0.7	0.022	0.072
GO:0046854	phosphatidylinositol phosphorylation	5	3	0.7	0.022	0.072
GO:0015672	monovalent inorganic cation transport	47	12	6.61	0.026	0.083
GO:0015698	inorganic anion transport	13	5	1.83	0.026	0.083
GO:0009165	nucleotide biosynthetic process	58	14	8.15	0.027	0.083
GO:1901293	nucleoside phosphate biosynthetic proces...	58	14	8.15	0.027	0.083
GO:0008272	sulfate transport	9	4	1.26	0.027	0.084
GO:0072348	sulfur compound transport	9	4	1.26	0.027	0.084
GO:0006644	phospholipid metabolic process	37	10	5.2	0.027	0.084
GO:0006811	ion transport	194	37	27.27	0.028	0.085
GO:0006182	cGMP biosynthetic process	6	3	0.84	0.040	0.103
GO:0006631	fatty acid metabolic process	6	3	0.84	0.040	0.103
GO:0016485	protein processing	6	3	0.84	0.040	0.103
GO:0033865	nucleoside bisphosphate metabolic proces...	6	3	0.84	0.040	0.103
GO:0033875	ribonucleoside bisphosphate metabolic pr...	6	3	0.84	0.040	0.103
GO:0034032	purine nucleoside bisphosphate metabolic...	6	3	0.84	0.040	0.103
GO:0046068	cGMP metabolic process	6	3	0.84	0.040	0.103
GO:0009116	nucleoside metabolic process	10	4	1.41	0.040	0.103
GO:0015988	energy coupled proton transmembrane tran...	10	4	1.41	0.040	0.103
GO:0015991	ATP hydrolysis coupled proton transport	10	4	1.41	0.040	0.103
GO:0019359	nicotinamide nucleotide biosynthetic pro...	10	4	1.41	0.040	0.103
GO:0019363	pyridine nucleotide biosynthetic process	10	4	1.41	0.040	0.103
GO:0033013	tetrapyrrole metabolic process	10	4	1.41	0.040	0.103
GO:0044262	cellular carbohydrate metabolic process	10	4	1.41	0.040	0.103
GO:0072525	pyridine-containing compound biosyntheti...	10	4	1.41	0.040	0.103
GO:0090662	ATP hydrolysis coupled transmembrane tra...	10	4	1.41	0.040	0.103
GO:0099131	ATP hydrolysis coupled ion transmembrane...	10	4	1.41	0.040	0.103
GO:0099132	ATP hydrolysis coupled cation transmembr...	10	4	1.41	0.040	0.103
GO:1901657	glycosyl compound metabolic process	10	4	1.41	0.040	0.103
GO:0090407	organophosphate biosynthetic process	84	18	11.81	0.040	0.103
GO:1901135	carbohydrate derivative metabolic proces...	144	28	20.24	0.042	0.106
module_8						
GO:0007186	G protein-coupled receptor signaling pat...	126	6	1.19	0.001	0.038
GO:0007165	signal transduction	327	8	3.09	0.009	0.103

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Table S4 – *Continued from previous page*

GO.ID	Term	Annotated	Significant	Expected	pvalue	FDR
GO:0007154	cell communication	334	8	3.16	0.010	0.103
GO:0023052	signaling	334	8	3.16	0.010	0.103
GO:0051716	cellular response to stimulus	384	8	3.63	0.023	0.182
GO:0050896	response to stimulus	409	8	3.87	0.032	0.214
GO:0065007	biological regulation	683	11	6.46	0.042	0.240
module_27						
GO:0043603	cellular amide metabolic process	175	67	13.29	<1e-30	4.0e-29
GO:0043604	amide biosynthetic process	166	65	12.6	< 1e-30	4.0e-29
GO:1901566	organonitrogen compound biosynthetic pro...	270	82	20.5	< 1e-30	4.0e-29
GO:0006518	peptide metabolic process	168	65	12.75	< 1e-30	4.0e-29
GO:0043043	peptide biosynthetic process	163	64	12.37	< 1e-30	4.0e-29
GO:0006412	translation	160	62	12.15	< 1e-30	4.0e-29
GO:0044271	cellular nitrogen compound biosynthetic ...	553	96	41.98	9.2e-18	1.9e-15
GO:0044249	cellular biosynthetic process	655	106	49.72	1.6e-17	1.9e-15
GO:1901576	organic substance biosynthetic process	664	106	50.41	4.7e-17	3.7e-15
GO:0009058	biosynthetic process	700	108	53.14	2.8e-16	1.6e-14
GO:0034645	cellular macromolecule biosynthetic proc...	544	91	41.3	1.4e-15	6.6e-14
GO:0009059	macromolecule biosynthetic process	546	91	41.45	1.8e-15	7.0e-14
GO:0044267	cellular protein metabolic process	574	88	43.57	1.3e-12	4.3e-11
GO:1901564	organonitrogen compound metabolic proces...	892	117	67.72	2.8e-12	8.2e-11
GO:0010467	gene expression	598	89	45.4	5.4e-12	1.4e-10
GO:0034641	cellular nitrogen compound metabolic pro...	872	110	66.2	3.4e-10	8.0e-09
GO:0019538	protein metabolic process	744	97	56.48	1.3e-09	2.8e-08
GO:0044237	cellular metabolic process	1425	150	108.18	2.3e-08	4.5e-07
GO:0044260	cellular macromolecule metabolic process	1029	115	78.12	2.5e-07	4.5e-06
GO:0006807	nitrogen compound metabolic process	1494	148	113.42	3.3e-06	5.5e-05
GO:0071704	organic substance metabolic process	1665	160	126.4	5.6e-06	8.7e-05
GO:1902600	proton transmembrane transport	25	10	1.9	6.3e-06	9.2e-05
GO:0044238	primary metabolic process	1584	153	120.25	1.0e-05	1.4e-4
GO:0009987	cellular process	1960	179	148.79	2.3e-05	3.0e-4
GO:0009141	nucleoside triphosphate metabolic proces...	27	9	2.05	1.0e-4	0.001
GO:0006575	cellular modified amino acid metabolic p...	8	5	0.61	1.1e-4	0.001
GO:0008152	metabolic process	2076	184	157.6	1.5e-4	0.002
GO:0042398	cellular modified amino acid biosyntheti...	5	4	0.38	1.5e-4	0.002
GO:0098655	cation transmembrane transport	36	10	2.73	2.3e-4	0.002
GO:0098660	inorganic ion transmembrane transport	36	10	2.73	2.3e-4	0.002
GO:0098662	inorganic cation transmembrane transport	36	10	2.73	2.3e-4	0.002
GO:0046034	ATP metabolic process	25	8	1.9	3.4e-4	0.003
GO:0043170	macromolecule metabolic process	1370	130	104	3.5e-4	0.003
GO:0009144	purine nucleoside triphosphate metabolic...	26	8	1.97	4.6e-4	0.004
GO:0009199	ribonucleoside triphosphate metabolic pr...	26	8	1.97	4.6e-4	0.004
GO:0009205	purine ribonucleoside triphosphate metab...	26	8	1.97	4.6e-4	0.004
GO:0009123	nucleoside monophosphate metabolic proce...	27	8	2.05	6.1e-4	0.004
GO:0009126	purine nucleoside monophosphate metaboli...	27	8	2.05	6.1e-4	0.004
GO:0009161	ribonucleoside monophosphate metabolic p...	27	8	2.05	6.1e-4	0.004
GO:0009167	purine ribonucleoside monophosphate meta...	27	8	2.05	6.1e-4	0.004
GO:0034220	ion transmembrane transport	41	10	3.11	7.2e-4	0.005
GO:0015985	energy coupled proton transport, down el...	11	5	0.84	7.7e-4	0.005
GO:0015986	ATP synthesis coupled proton transport	11	5	0.84	7.7e-4	0.005
GO:1901137	carbohydrate derivative biosynthetic pro...	76	14	5.77	0.001	0.009
GO:0045333	cellular respiration	8	4	0.61	0.002	0.011
GO:0006754	ATP biosynthetic process	19	6	1.44	0.002	0.011
GO:0009142	nucleoside triphosphate biosynthetic pro...	19	6	1.44	0.002	0.011
GO:0009145	purine nucleoside triphosphate biosynthe...	19	6	1.44	0.002	0.011
GO:0009201	ribonucleoside triphosphate biosynthetic...	19	6	1.44	0.002	0.011
GO:0009206	purine ribonucleoside triphosphate biosy...	19	6	1.44	0.002	0.011
GO:0015672	monovalent inorganic cation transport	47	10	3.57	0.002	0.011
GO:0006790	sulfur compound metabolic process	14	5	1.06	0.003	0.014
GO:0006091	generation of precursor metabolites and ...	20	6	1.52	0.003	0.014
GO:0015980	energy derivation by oxidation of organi...	9	4	0.68	0.003	0.014
GO:0061024	membrane organization	9	4	0.68	0.003	0.014
GO:0009150	purine ribonucleotide metabolic process	42	9	3.19	0.004	0.016
GO:0009259	ribonucleotide metabolic process	42	9	3.19	0.004	0.016
GO:0009124	nucleoside monophosphate biosynthetic pr...	21	6	1.59	0.004	0.016

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Table S4 – *Continued from previous page*

GO.ID	Term	Annotated	Significant	Expected	pvalue	FDR
GO:0009127	purine nucleoside monophosphate biosynth...	21	6	1.59	0.004	0.016
GO:0009156	ribonucleoside monophosphate biosyntheti...	21	6	1.59	0.004	0.016
GO:0009168	purine ribonucleoside monophosphate bios...	21	6	1.59	0.004	0.016
GO:0007005	mitochondrion organization	10	4	0.76	0.005	0.018
GO:0015988	energy coupled proton transmembrane tran...	10	4	0.76	0.005	0.018
GO:0015991	ATP hydrolysis coupled proton transport	10	4	0.76	0.005	0.018
GO:0090662	ATP hydrolysis coupled transmembrane tra...	10	4	0.76	0.005	0.018
GO:0099131	ATP hydrolysis coupled ion transmembrane...	10	4	0.76	0.005	0.018
GO:0099132	ATP hydrolysis coupled cation transmembr...	10	4	0.76	0.005	0.018
GO:0019693	ribose phosphate metabolic process	44	9	3.34	0.005	0.018
GO:0009100	glycoprotein metabolic process	29	7	2.2	0.005	0.018
GO:0006163	purine nucleotide metabolic process	46	9	3.49	0.007	0.024
GO:0072521	purine-containing compound metabolic pro...	47	9	3.57	0.008	0.028
GO:0009056	catabolic process	82	13	6.22	0.008	0.028
GO:1901565	organonitrogen compound catabolic proces...	49	9	3.72	0.010	0.034
GO:0009152	purine ribonucleotide biosynthetic proce...	33	7	2.51	0.010	0.034
GO:0009260	ribonucleotide biosynthetic process	33	7	2.51	0.010	0.034
GO:0046390	ribose phosphate biosynthetic process	33	7	2.51	0.010	0.034
GO:1901575	organic substance catabolic process	76	12	5.77	0.011	0.036
GO:1901135	carbohydrate derivative metabolic proces...	144	19	10.93	0.011	0.037
GO:0022900	electron transport chain	7	3	0.53	0.012	0.039
GO:0006486	protein glycosylation	28	6	2.13	0.016	0.050
GO:0009101	glycoprotein biosynthetic process	28	6	2.13	0.016	0.050
GO:0043413	macromolecule glycosylation	28	6	2.13	0.016	0.050
GO:0070085	glycosylation	28	6	2.13	0.016	0.050
GO:0006352	DNA-templated transcription, initiation	21	5	1.59	0.018	0.053
GO:0007015	actin filament organization	8	3	0.61	0.018	0.053
GO:0031503	protein-containing complex localization	8	3	0.61	0.018	0.053
GO:0044248	cellular catabolic process	72	11	5.47	0.018	0.053
GO:0006164	purine nucleotide biosynthetic process	37	7	2.81	0.019	0.055
GO:0030163	protein catabolic process	38	7	2.88	0.022	0.061
GO:0072522	purine-containing compound biosynthetic ...	38	7	2.88	0.022	0.061
GO:0051188	cofactor biosynthetic process	30	6	2.28	0.023	0.063
GO:0009057	macromolecule catabolic process	50	8	3.8	0.033	0.088
GO:0055085	transmembrane transport	283	30	21.48	0.034	0.092
GO:0048518	positive regulation of biological proces...	17	4	1.29	0.035	0.093
GO:0016043	cellular component organization	162	19	12.3	0.035	0.093
GO:0051186	cofactor metabolic process	42	7	3.19	0.036	0.094
GO:0006753	nucleoside phosphate metabolic process	72	10	5.47	0.043	0.109
GO:0006812	cation transport	92	12	6.98	0.043	0.109
GO:0030029	actin filament-based process	11	3	0.84	0.045	0.109
GO:0030036	actin cytoskeleton organization	11	3	0.84	0.045	0.109
GO:0019725	cellular homeostasis	35	6	2.66	0.045	0.109
GO:0044257	cellular protein catabolic process	35	6	2.66	0.045	0.109
GO:0051603	proteolysis involved in cellular protein...	35	6	2.66	0.045	0.109
GO:0071840	cellular component organization or bioge...	190	21	14.42	0.049	0.116

Table S5: Genes with strongest increase in connectivity in module 27.

	C. obscurior gene	*D. melanogaster* ortholog	pfam domains	putative function
1	Cobs_15828	Protein lifeguard 4 (Q9DA39)	Bax1-I	Anti-apoptotic protein aka Golgi anti-apoptotic protein (GAAP)
2	Cobs_06161	FBgn0011284	RS4NT, S4, Ribosomal_S4e, KOW, 40S_S4_C	Ribosomal protein S4
3	Cobs_09326	FBgn0015756	Ribosomal_L6	Ribosomal protein L9
4	Cobs_18136	FBgn0014391	ATP-synt_Eps	ATP synthase epsilon chain
5	Cobs_12509	FBgn0261596	Ribosomal_S24e	Ribosomal protein S24
6	Cobs_17251	Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial (P45954)	Acyl-CoA_dh_N, Acyl-CoA_dh_M, Acyl-CoA_dh_1, Linker_histone, adh_short	short/branched chain specific acyl-CoA dehydrogenase, mitochondrial-like
7	Cobs_17813	FBgn0015031	COX6C	cytochrome c oxidase subunit VIc
8	Cobs_07556	FBgn0031059	NA	uncharacterised
9	Cobs_07129	60S ribosomal protein L35 (Q3MHM7)	Ribosomal_L29	60S ribosomal protein L35
10	Cobs_00057	26S proteasome complex subunit SEM1 (P60897)	DSS1_SEM1	26S proteasome complex subunit DSS1