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Elevated expression of ageing and immunity genes in queens of the black garden ant

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Abstract

Studies in model organisms have identified a variety of genes whose expression can be experimentally modulated to produce changes in longevity, but whether these genes are the same as those involved in natural variation in lifespan remains unclear. Social insects boast some of the largest lifespan differences known between plastic phenotypes, with queen and worker lifespans differing by an order of magnitude despite no systematic nucleotide sequence differences between them. The contrasting lifespans of queens and workers are thus the result of differences in gene expression. We used RNA sequencing of brains and legs in 1-day-old and 2-month-old individuals of the ant *Lasius niger* to determine whether genes with queen-biased expression are enriched for genes linked to ageing in model organisms. Because the great longevity of queens may require investment into immune processes, we also investigated whether queen-biased genes are enriched for genes with known roles in immunity. Queen-biased genes in legs were enriched for ageing genes and for genes associated with increasing rather than decreasing lifespan. Queen-biased genes in legs were also enriched for immune genes, but only in 1-day-old individuals, perhaps linked to the changing roles of workers with age. Intriguingly, the single most differentially expressed gene between 1-day-old queen and worker brains was an extra-cellular form of CuZn Superoxide Dismutase (SOD3), raising the possibility of an important role of anti-oxidant genes in modulating lifespan.

1 Introduction

The expected lifespan of an organism in the absence of extrinsic mortality is not rigidly determined by its genome, but instead can vary plastically within a species (Fielenbach & Antebi 2008, Lucas & Keller 2017). In some taxa, naturally-occurring environmentally-determined polymorphisms can be associated with large differences in lifespan (Flatt et al. 2013), yet the genes involved in this natural plastic variation in many cases remain unknown. Studies in model organisms have revealed many genes whose expression can be experimentally modulated to affect lifespan in the laboratory (Tacutu et al. 2013), but it remains unknown whether these genes are also involved in plastic longevity differences in the wild.

A striking example of natural plastic polymorphisms in longevity is found in advanced social insects such as ants, where females can develop into either queens, which are typically very long-lived (Keller & Genoud 1997), or workers, which have a shorter lifespan. In most species, queens and workers share a common genome and their different lifespans are therefore regulated through differential gene expression (Schwander et al. 2010). The origins of these expression differences can be due to a range of factors, including epigenetic variation and physiological reactions to different environments. The naturally-occurring polymorphisms in social insects have thus been the focus of study to understand the basis of these plastic differences in longevity and the changes that accompany ageing (Aurori et al. 2014, Jemielity et al. 2007, Lucas & Keller 2017, Lucas & Keller 2014, de Verges & Nehring 2016). Gene expression studies have for the most part focused on a few candidate pathways (Aamodt 2009, Corona et al. 2005, Corona et al. 2007, Lucas et al. 2016, Parker et al. 2004a) or used transcriptome-wide analysis (Seehuus et al. 2013) but have not systematically investigated the extent of overlap with genes involved in ageing in model organisms.

In this study, we first test the hypothesis that genes related to ageing in model organisms (list

obtained from the *GenAge* database, Tacutu et al. 2013) also underlie the large-scale natural plasticity in longevity in the black garden ant *Lasius niger*. Queens of *L. niger* are substantially larger than workers, physiologically specialised for egg-laying, and live up to 30 years (Hölldobler & Wilson 1990), as compared to only 3 years for workers (Kramer et al. 2016). After emergence as adults, queens spend a few weeks accumulating nutritional resources before engaging in a mating flight, after which successfully-mated queens found an incipient colony, using their nutritional reserves to feed their first cohort of workers (Hölldobler & Wilson 1990). Once the first workers are produced, queens stop feeding the brood and become dedicated egg-layers. Workers conduct all the other colony tasks.

Longevity can be affected by investment in the immune system, with higher investment contributing to reduced risk of infection, but carrying costs in the form of energetic demands (Moret & Schmid-Hempel 2000). The benefit of down-regulating the immune system may therefore depend on the resources available and the environmental hostility (Schmid-Hempel 2005). In laboratory-reared *Drosophila,* down-regulation of immune gene expression was found in lines with extended longevity (Carnes et al. 2015), but this may be due to the lack of immune threats in a laboratory setting. Whether the long life of social insect queens is linked to changes in immunity gene expression remains unknown. In honeybees, results suggest that worker pupae have higher levels of Prophenoloxidase (PPO) expression than queen pupae (Lourenço et al. 2005) and adult queens may have higher Phenoloxidase (PO) activity than workers (Schmid et al. 2008). PPO is the molecular precursor to PO, which regulates the melanisation response, an important aspect of the insect immune system (González-Santoyo & Córdoba-Aguilar 2012). However, insect immunity is a multi-faceted system involving several pathways of humoral and cellular responses (Lemaitre & Hoffmann 2007). Instead of focusing on a single pathway, we therefore investigate whether queens show increased expression of genes linked to immunity by globally studying the expression of genes obtained from an extensive database of insect immunity genes (Brucker et al. 2012).

These targeted analyses of genes linked to ageing and immunity investigate whether the longevity of queens is associated with a concerted shift in the expression of a large group of genes. Because individual genes may also play crucial roles in queen longevity, we also identified the genes that show the strongest patterns of differential expression between queens and workers.

To perform these analyses of gene expression, we chose two tissues. First, we chose the brain because it affects many traits linked to survival and organismal function. Second, we chose legs as they mostly comprise muscles that perform similar tasks in queens and workers, and should thus allow us to identify genes whose expression is inherently different between the two castes, rather than genes associated with these different roles. We measured gene expression in 1-day-old and 2-month-old individuals to represent two divergent points in development. One-day-old queens and workers have recently emerged from the pupal stage and are thus very young adults. By contrast, 2-month-old workers are fully developed and conduct typical worker tasks. Queens of this age are also in a "typical" physiological state where they have initiated egg production. We did not use older individuals in order to avoid confounding the differences between castes with those caused by differential rates of ageing in queens and workers. Using an age at which workers could be considered to be old while queens are still young would make it impossible to differentiate the causes of differential ageing from its consequences.

2 Results

Overall, substantially fewer genes were significantly differentially-expressed between queens and worker in brains than in legs. Out of the 63,661 transcriptome components, 1,384 (2.2%) were differentially expressed in 1-day-old brains and 486 (0.8%) were differentially expressed in 2-month-old brains. By contrast, these values were 5,792 (9.1%) in 1-day-old legs and 10,400 (16.3%) in 2-month-old legs (Table 1). Furthermore, differentially-expressed genes in brains were

more frequently queen-biased than worker-biased (binomial tests, 1-day-old: $P < 0.0001$, 2-month-old: $P < 0.0001$), while in legs they were more frequently worker-biased than queen-biased (binomial test, 1-day-old: $P < 0.0001$, 2-month-old: $P < 0.0001$; Table 1).

2.1 Differential expression of ageing genes

A total of 429 homologs of the aging genes listed in the *GenAge* database (Tacutu et al. 2013) were identified in *L. niger* by strict reciprocal blast. In each of the four age / tissue combinations (1-day-old legs, 1-day-old brains, 2-month-old legs, 2-month old brains), there were proportionally more ageing genes among queen-biased genes than among worker-biased genes (Table 1, Supplementary Figure S1), but the difference was significant only in the legs of 1-day-old individuals (Fisher's exact test; 1-day-old legs: $P < 0.0001$; 2-month-old legs: $P = 0.58$, 1-day-old brains: $P = 0.47$, 2-month-old brains: $P = 0.58$).

Out of the 2651 genes that were queen-biased in 1-day-old legs, 96 (3.6%) were ageing genes, as compared to 17 out of 1009 (1.7%) in 1-day-old brains, 71 out of 4261 (1.7%) in 2-month-old legs and four out of 356 (1.1%) in 2-month-old brains. These differences were significant between 1-day-old legs and each of the other three categories (Fisher's exact test vs. 1-day-old brains: $P = 0.0018$, vs. 2-month-old legs: $P < 0.0001$, vs. 2-month-old brains: $P = 0.011$). Out of 6139 genes that were worker-biased in 2-month-old legs, 93 (1.5%) were ageing genes, as compared to four out of 375 (1.1%) in 1-day-old brains, 34 out of 3141 (1.1%) in 1-day-old legs and 0 out of 130 (0%) in 2-month-old brains. None of these differences were significant.

The *GenAge* database provides information on whether increasing a gene's expression is associated with increased lifespan (“pro-longevity”) or decreased lifespan (“anti-longevity”). In legs, the ratio of pro-longevity to anti-longevity genes was higher in queen-biased genes than in worker-biased genes both in 1-day-old and 2-month-old individuals (Table 2), although the difference was only

significant in 2-month-old individuals (Fisher's exact test, 1-day-old: P = 0.4, 2-month-old: P = 0.036). In brains, there was little statistical power because there were only very few worker-biased genes (Table 2).

Using a less strict reciprocal blast, homologs of a further 308 genes from the *GenAge* database were identified in *L. niger*, leading to a total of 737 genes. Of these, 24 were consistently worker-biased and 25 were consistently queen-biased within an age or tissue (Supplementary Data S2), compared to 1145 and 475 non-ageing genes that were consistently worker- and queen-biased respectively. The consistently queen-biased ageing genes include two anti-oxidant genes that showed consistent queen-biased expression (Peroxiredoxin 1 / Thioredoxin Peroxidase 1 was queen-biased in both legs and brains of 1-day-old individuals; Glutathione Peroxidase 2 was queen-biased in both legs and brains of 2-month-old individuals, Supplementary Data S2).

2.2 Differential expression of immunity genes

A total of 86 homologs of the immunity genes listed in the Insect Innate Immunity Database (IIID) (Brucker et al. 2012), were identified in *L. niger* by strict reciprocal blast. In 1-day-old legs, 1-day-old brains and 2-month-old legs, there were proportionally more immunity genes among queen-biased genes than among worker-biased genes (Table 1, Supplementary Figure S2), the difference being significant in 1-day-old legs (Fisher's exact test; 1-day-old legs: P = 0.0007; 1-day-old brains: P = 0.2; 2-month-old legs: P = 0.31). In 2-month-old brains, there was no difference in the proportion of immunity genes between queen-biased and worker-biased genes (Fisher's exact test; P = 1).

Out of the 356 genes that were queen-biased in 2-month-old brains, five (1.4%) were immunity genes, as compared to 11 out of 1009 (1.1%) in 1-day-old brains, 23 out of 2651 (0.9%) in 1-day-old legs and 18 out of 4261 (0.4%) in 2-month-old legs. These differences were only significant

between 2-month-old legs and the other three categories (Fisher's exact test vs. 2-month-old brains: $P = 0.028$, vs. 1-day-old legs: $P = 0.024$, vs. 1-day-old brains: $P = 0.016$). Out of the 130 worker-biased genes in 2-month-old brains, two (1.5%) were immunity genes, as compared to 18 out of 6139 (0.3%) in 2-month-old legs, one out of 375 (0.3%) in 1-day-old brains and seven out of 3141 (0.2%) in 1-day-old legs. Only the difference between 2-month-old brains and 1-day-old legs was significant ($P = 0.047$).

Using a less strict reciprocal blast, homologs of a further 72 genes from the IIID database were identified in *L. niger*, leading to a total of 158 genes. Of these, only four were consistently worker-biased while 23 were consistently queen-biased within an age or tissue (Supplementary Data S3), compared to 1165 and 477 non-immunity genes that were consistently worker- and queen-biased respectively. The consistently queen-biased genes included Pro-phenoloxidase (PPO), which was queen-biased in the legs of both 1-day-old and 2-month-old individuals.

2.3 Genes showing the strongest patterns of differential expression

We identified the genes that showed the strongest statistical support for caste-biased expression in each tissue, either considering 1-day-old and 2-month-old individuals separately or together. These genes are listed in Supplementary Data S1, and we highlight three of these genes below.

2.3.1 CuZn-SOD

In the combined analysis of 1-day-old and 2-month-old individuals, the most significantly differentially-expressed gene between queens and workers in brains was the anti-oxidant enzyme extra-cellular CuZn Superoxide Dismutase (SOD3, see Supplementary Information for details of annotation). In legs, the expression of this gene was also queen-biased in 2-month-old individuals (FDR-adjusted P-value, $Q = 0.002$), but was worker-biased in 1-day-old individuals ($Q < 0.0001$; Fig. 1A).

A previous study of SOD in *L. niger* found no difference in expression between queens and workers (Parker et al. 2004a). However, that study focused on the cytosolic CuZn-SOD (SOD1), while our results pertain to SOD3. To confirm that the different results were due to the different gene under consideration rather than different experimental conditions, we identified the homolog of SOD1 in our *L. niger* transcriptome by blasting the published sequence for *L. niger* SOD1 (accession AY309973) against the transcriptome. This confirmed that SOD1 was not differentially-expressed between queens and workers in any of the age / tissue combinations (Supplementary Figure S3).

2.3.2 Insulin-like peptide

In the combined analysis of 1-day-old and 2-month-old individuals, Probable Insulin-Like Peptide 1 was amongst the top five most differentially expressed genes in brains. Expression of this gene was significantly greater in queens than workers in brains of both ages ($Q < 0.0001$, Fig. 1B). In legs, expression was substantially lower than in brains (FPKM range 1-8 in legs compared to 14-62 in brains) and was queen-biased in 1-day-old individuals ($Q = 0.038$) but worker-biased in 2-month-old individuals ($Q < 0.0001$, Fig. 1B).

2.3.3 Fatty acid synthase

Fatty acid synthase was amongst the five most differentially expressed genes in the legs of 2-month-old individuals, with greater expression in queens than workers ($Q < 0.0001$). This gene showed very low expression in all age / tissue / caste combinations except in the legs of 2-month-old queens (Supplementary Figure S4).

3 Discussion

In 1-day-old legs, the proportion of ageing genes was higher among queen-biased genes than

worker-biased genes. Also, in 2-month-old legs there was a higher ratio of pro-longevity to anti-longevity genes in queen-biased than worker-biased genes (these genes are listed in Supplementary Data S5). The same patterns were found in other tissue/age combinations, although they were not significant. This enrichment of genes from the *GenAge* database among queen-biased genes indicates an overlap in the physiological pathways that underlie variation in longevity in model organisms and those that regulate the 10-fold difference in longevity between queen and worker ants. Differential expression of ageing genes between 1-day-old queens and workers could influence longevity by sowing the early seeds of senescence, for example by affecting the early accumulation of somatic damage. These findings support the notion that results of experimental ageing research in model organisms can inform our understanding of the evolution of ageing by natural selection. Conversely, and perhaps more importantly, results from social insects are relevant to understanding how the lifespan of an organism can be modified through experimental intervention.

Genes linked to immunity were in general more likely to be queen-biased than worker-biased, the difference being significant only in the legs of 1-day-old individuals. These results contrast with results from *Drosophila*, where down-regulation of immune response genes was found in selection lines with extended lifespan (Carnes et al. 2015). One possible explanation for this apparent discrepancy is that there are probably costs for higher expression of immunity genes in terms of energy and trade-offs with decreased immune investment leading to an improvement of other important functions, thus extending longevity in the absence of disease (Moret & Schmid-Hempel 2000, Schmid-Hempel 2005). However, in the wild, longer-lived individuals may benefit from investing more into immune genes to better cope with immune threats, even if this induces some cost that would reduce lifespan under conditions without disease. A possible explanation for the lack of significant differences in immune gene expression between queens and workers in 2-month-old individuals is that 2-month-old queens are being cared for by their workers and thus benefit

from protection through social immunity (Cremer et al. 2007), reducing their exposure to pathogens.

There are two limitations to our study. The first is that brains and legs are not crucially involved in immunity. It would thus be interesting to conduct a study to investigate whether similar, or more marked, difference are found in the level of expression of immune genes in the fat body and hemolymph of queens and workers. The second limitation is that we only analysed two age stages. It would be interesting to determine whether ageing and immunity genes are also more highly expressed in queens than workers in the larval and pupal stages, where important developmental processes occur, as well as in later life. A particular challenge to studying older queens and workers will be to disentangle the effects of ageing from its causes. Using many different age stages of both castes would allow the accurate measurement of age-trajectories for gene expression and identify genes differently expressed between queens and workers without being due only to an accelerated age trajectory.

PPO was queen-biased in the legs of both 1-day-old and 2-month-old individuals. PPO is the molecular precursor to PO, which plays an important role in the melanisation response in insect immune pathways (González-Santoyo & Córdoba-Aguilar 2012). In a previous publication (Lucas et al. 2017a) we reported that queens showed higher expression of Spn27A, which inhibits the conversion of PPO to PO and argued that this was unlikely to reflect reduced levels of immunity in queens, hypothesising that queens might have higher levels of PPO than workers and thus require high levels of Spn27A to prevent it from forming PO while there is no active immune threat. Our finding that queens show higher expression of PPO than workers supports this hypothesis. The up-regulation of PPO in queens is consistent with results in the ant *Formica exsecta*, where PPO expression is up-regulated in queens compared to males (Stucki et al. 2017). Like workers, males are shorter-lived than queens, suggesting that reduced expression of PPO in short-lived phenotypes

is not restricted to workers.

Several genes linked to resistance against oxidative stress showed queen-biased expression. Strikingly, an extra-cellular CuZn-SOD (SOD3, Parker et al. 2004b) was the most highly significant queen-biased gene in a combined analysis of brains of 1-day-old and 2-month-old queens and workers. While extracellular CuZn SOD has been linked to ageing in mammals, it is a rarely-studied form of CuZn-SOD in insects (Blackney et al. 2014, Favrin et al. 2013, Jung et al. 2011), in contrast to its cytosolic counterpart SOD1, which has been the focus of expression studies (Aurori et al 2014, Grozinger et al. 2007, Parker et al. 2004a, Parkes et al. 1998). The expression of SOD1 does not differ between queens and workers in *L. niger* (Parker et al. 2004a and our data). By contrast, in honeybees, SOD1 shows queen-biased expression in the brain (Grozinger et al. 2007). Our results reconcile these findings, revealing that ants also have queen-biased expression of CuZn-SOD in the brain, albeit in a different form, lending fresh credence to the potential role of increased anti-oxidant expression in the exceptional lifespans of ant queens. The role of SOD3 in queen longevity may be more important in the brain than in legs since expression levels were much lower and expression was not consistently queen-biased in legs. In *Drosophila*, disruption of SOD3 expression has produced conflicting results, with one study reporting a negative effect on lifespan (Jung et al. 2011) while two more reported no significant effect (Blackney et al. 2014, Favrin et al. 2013). A valuable study would be to over-express SOD3 in *Drosophila* brains to determine its impact on longevity.

Another possible role of SOD3 in the longevity difference between queens and workers is through its role in immunity. In leaf beetles, knockdown of SOD3 by RNAi does not significantly affect lifespan but reduces survival after fungal infection (Gretscher et al. 2016). Furthermore, insect SOD3 has been found to inhibit PO activity in *Drosophila* hemolymph (Colinet et al. 2011), and may thus also play a role in keeping the high levels of PO in check in ant queens.

Two additional antioxidant genes, Peroxiredoxin 1 and Glutathione Peroxidase 2, were consistently queen-biased in legs and brains of 1-day-old individuals (Peroxiredoxin 1) and 2-month-old individuals (Glutathione Peroxidase 2). The links between anti-oxidants and ageing have been actively studied in social insects (Aurori et al. 2014, Parker et al. 2004b,a), and while their importance in explaining longevity in general has been questioned (Pérez et al. 2009, Parker et al. 2004a), our results suggest that their role should not yet be completely dismissed. The picture concerning the difference in longevity between queens and workers has in fact become increasingly interesting. Compared to workers, *L. niger* queens show increased expression of anti-oxidant genes (this study) and increased expression of DNA and protein repair genes (Lucas et al. 2016), yet a recent study found no evidence of differential rates of DNA or protein damage accumulation with age (Lucas et al. 2017b). This latter study measured the levels of DNA strand-breaks and found that while these increased with age, this increase was either greater in queens (heads) or similar in queens and workers (legs). One way of reconciling these results is if a different form of damage, that does not manifest as strand breaks, accumulates faster in workers than in queens. For example, oxidative DNA damage can disrupt transcription and cause the production of aberrant mRNA transcripts (Brégeon & Doetsch 2011). Alternatively, since queens show signs of immune gene up-regulation compared to workers, these antioxidants may be up-regulated as a response to the oxidative cytotoxicity associated with elevated immune function (Nappi & Christensen 2005).

We found that the homolog of a *Drosophila* probable Insulin-like peptide (ILP) was overexpressed in queen brains compared to worker brains. ILPs play a central role in the Insulin–IGF-1 Signaling (IIS) pathway, which is an evolutionarily-conserved nutritionally-sensitive regulator of growth, fertility and longevity (Partridge et al. 2011). Since queens typically receive higher levels of nutrition than workers, it makes sense for nutrition-associated proteins such as ILPs to be up-regulated in this caste. In honeybees, ILP expression is higher in old winter bees than in young winter bees (Aurori et al. 2014), possibly indicating inherent changes in gene expression with age.

Similar results to ours were obtained in other ants, where the expression of IIS genes is also higher in adult queens than workers (Lu & Pietrantonio 2011, Okada et al. 2010). In these studies, expression was compared in whole bodies (Lu & Pietrantonio 2011) and abdomens (Okada et al. 2010), where the ovaries reside. Including ovary tissue in this way may reveal patterns directly linked to reproduction rather than to more general caste polymorphisms. Our results show that even in tissues not directly-linked to reproduction, a gene from the IIS pathway is amongst the most strongly queen-biased genes in the adult transcriptional profile.

We also found that the expression of a fatty-acid synthase was highly queen-biased in the legs of 2-month-old individuals. A fatty-acid synthase homolog has previously been shown to be more highly expressed in queens than workers in the ant *Harpegnathus saltator* (Bonasio et al. 2010). Interestingly, fatty acid synthase was very lowly expressed in nearly all age / tissue / caste combinations, with the exception of the legs of 2-month-old queens, suggesting an as yet unidentified age- and caste-specific role.

In summary, our results provide insights into the age- and tissue-specific gene expression differences between highly-differentiated plastic queen and worker phenotypes. Our targeted analysis of ageing genes highlights parallels between the genes involved in experimentally manipulating lifespan in model organisms and those that underlie evolved differences in longevity. This queen-biased expression of ageing genes is accompanied by queen-biased expression of immunity genes at least in the first few days of adulthood, which may play a role in ensuring the early survival of queens in the face of immune challenges. The agnostic study of highly differentially-expressed genes highlights nutrition and defence against oxidative stress as important pathways that differentiate queens and workers, likely to be linked to their different diets and longevity, and revives the notion that anti-oxidants play a key role in the striking longevity of social insect queens. Overall, our results reflect the multiple processes involved in ageing (immunity,

nutrition, resistance to oxidative stress), indicating that extended longevity is achieved by the modulation of multiple different processes in parallel.

4 Methods

4.1 Sample collection

The rearing and collection methods used in this study have been previously described in Lucas et al. (2016). All samples were collected from the campus of the University of Lausanne, Switzerland. Briefly, we collected queen and worker pupae, along with mature workers, from the field to establish queenless laboratory colonies, where the mature workers then reared the pupae to adulthood. 1-day-old queens and workers were obtained each day from these queenless laboratory colonies by freezing newly-emerged individuals into liquid nitrogen.

To obtain 2-month-old workers, worker pupae (originally collected from the field as part of the queenless colonies) were transferred from laboratory queenless colonies into laboratory queenright colonies established from the mating flight the previous year, and in which existing adult workers had been marked with spots of paint on the abdomen. Forty worker pupae were transferred into each queenright colony, and were then checked every 3-4 days to monitor their emergence as adults. The average date between first and last emergence within a colony was used as day 0 for age estimation. First and last emergence differed from the mean by at least two days and at most nine days. One week after the last transferred pupae had emerged from a colony, all pre-existing workers were removed, leaving only the queen and the introduced workers. To prevent the subsequent emergence of workers that were not from the transferred pupae, large larvae and pupae were regularly removed from the queenright colonies. Workers were killed by freezing in liquid nitrogen two months after emergence.

To obtain 2-month-old queens, queens were collected during the mating flight and brought to the

laboratory where they were allowed to establish a colony. Queens were then killed by freezing in liquid nitrogen seven weeks after the mating flight, approximately one week after the emergence of the first workers. Queens were assumed to be two weeks old at the point of the mating flight, and were therefore around 9 weeks old on the day of final collection.

The inaccuracy in estimating the age of 2-month-old individuals did not result in increased variation in gene expression compared to 1-day-old individuals. In fact, variation was greater in 1-day-old than 2-month-old individuals, as has been previously discussed (Lucas et al. 2017a). All the samples collected here spent their larval development in the field and were being kept in the laboratory at the time of their final collection. The 2-month-old queens differ from the other samples in that they completed their pupal development in the field, as well as the first few weeks of adult life. Since queens cannot be mated in the laboratory in this species, it was not possible to raise them in the laboratory and thereby perfectly match their history to that of the workers. However, they were in the laboratory for seven weeks before collection, thus minimising an environmental effect on gene expression.

2-month-old queens were collected from the mating flight, making it highly unlikely that any two individuals were from the same colony. For each other age / tissue / caste combination, individuals from different colonies were used for each replicate, so replicates were also independent. Where possible, samples of different caste and age were matched for colony of origin (Supplementary Data S4). The samples presented here are the same as those used in a previous study (Lucas et al. 2016).

For legs, six independent replicates were obtained for each age / caste combination. Legs were separated from the thorax on dry ice. For each worker replicate, the legs of ten workers were pooled. For ten of the queen replicates, the legs of five queens were pooled. The two remaining queen replicates used the pooled legs from four and three queens respectively.

For brains, five independent replicates were obtained for each age / tissue / caste combination.

359 Brains were dissected out in PBS (Sigma, pH 7.2-7.4) chilled on ice and were immediately
360 transferred into TRIZOL and stored at -80°C. Each replicate consisted of a pool of six workers
361 brains or four queens brains.

362 4.2 RNA extraction and sequencing

363 The RNA extraction and sequencing for these samples are as detailed in Lucas et al. (2016). RNA
364 was extracted using TRIZOL. Library preparation and sequencing were performed at the Lausanne
365 Genomic Technologies Facility, Centre for Integrative Genomics, University of Lausanne,
366 Switzerland. Strand-specific libraries were prepared using the Illumina TruSeq Stranded mRNA
367 reagent kit (Illumina, San Diego, CA) and sequenced by Illumina HiSeq 2000/2500 to obtain 100
368 nucleotide paired-end reads. Raw sequencing reads are available in the NCBI Short Read Archive
369 (accession numbers: SRP069113).

370 The 24 libraries obtained from legs were divided into two groups of 12, with each group consisting
371 of all caste / age combinations from three of the six replicates. Each group was sequenced on two
372 lanes of the Illumina platform.

373 The 20 libraries obtained from brains were sequenced together on four lanes of the Illumina
374 platform. Three of these lanes were revealed to be under-loaded and the libraries were therefore
375 sequenced on a further three lanes. Data from all seven lanes were combined in the analysis.

376 4.3 Gene expression analysis

377 Reads were aligned using Bowtie2 (Langmead & Salzberg 2012) to the *L. niger* transcriptome
378 described in (Lucas et al. 2016). Upon submission of the transcriptome to NCBI, 57 out of 63,718
379 components were identified as potentially being a result of contamination. These components were
380 removed from the analysis, leaving 63,661 components. Reads aligning to transcripts within the
381 same isogroup were combined to obtain a single alignment read count per gene. Combined read
382 counts were analysed using *edgeR* (Robinson et al. 2010) using normalisation by Trimmed Mean of

M-values (Robinson & Oshlack 2010). The effects of caste on gene expression were calculated using generalised linear modelling with caste, age and handling batch (samples that were handled together in the laboratory) included as categorical fixed effects. The R package *fdrtool* was used to calculate false discovery rate (FRD) adjusted *P* values (*Q* values) and a threshold *Q* value of 0.05 was used to identify significantly differentially-expressed genes. Legs and brains were analysed separately.

We identified the genes that showed the most strongly significant (lowest *P* value) differential expression between castes in 1-day-old individuals, 2-month-old individuals or in a combined analysis of both age groups (Supplementary Data S1). This analysis was also repeated after filtering for genes with a fold change of at least 2 (log2-fold change > 1). Three genes identified in this way (a homolog of Probable ILP-1, and two genes with homology to Locusta Insulin-Related Peptides) had highly similar expression profiles (Fig. 1B and Supplementary Fig. S5), suggesting that they may be the same gene incorrectly assembled into three different isogroups. We therefore discuss these as a single gene.

4.4 Sequence annotation

Annotation of the most differentially expressed genes (Supplementary Data S1) was performed using BLASTs (Altschul et al. 1997) performed with a e-value cut-off of 10^{-4}. Open Reading Frames (ORF) were predicted with Augustus (v2.5.5; (Stanke & Waack 2003) using the honeybee as a model for gene structure. Sequences of interest in which an ORF was detected were annotated by reciprocal BLAST: a given contig in the transcriptome was accepted as a homolog for a known protein if the protein was the top blastp hit of the contig's predicted protein sequence against the Swissprot proteome, and the predicted protein sequence was a blastp hit of this protein against all translated ORFs in the transcriptome. This method was initially performed against the *Drosophila melanogaster* Swissprot proteome. If no suitable gene was found, it was repeated with the human

Swissprot and then *Apis mellifera* Swissprot/Trembl proteomes.

For the sequences that did not contain any ORFs or for which the above method was not able to produce an annotation, we report the top translated BLAST (blastx) hit of the nucleotide sequence. This BLAST was run against the *D. melanogaster* Swissprot proteome. If no hit was obtained, it was repeated with the human Swissprot and then *A. mellifera* Swissprot/Trembl proteomes. Finally, for sequences that could not be annotated using either of the above methods, we report the top blastn hit of the nucleotide sequence against the non-redundant NCBI database (nr/nt). Of the 27 genes that were annotated, 13 were annotated by reciprocal BLAST of the ORF, 3 were annotated by translated BLAST, and 11 were annotated by blastn to nr/nt.

4.5 Candidate genes linked to ageing and immunity.

A list of genes with demonstrated links to ageing in model organisms was downloaded from the *GenAge* database (Tacutu et al. 2013, http://genomics.senescence.info/genes/models_genes.zip) on 02/12/2016 (henceforth referred to as "ageing genes"). A fasta file of proteins playing a role in insect immunity was downloaded from the Insect Innate Immunity Database (Brucker et al. 2012), http://www.vanderbilt.edu/IIID) on 25/02/17 by searching the database with no search queries in order to obtain the full list of genes (henceforth referred to as "immunity genes"). Orthologs of these genes in the *L. niger* transcriptome were obtained by reciprocal blast. The protein sequences for each ageing and immunity gene was blasted (tblastn) against the *L. niger* transcriptome. The top hit was then blasted (blastx) back against the NCBI non-redundant protein database (nr) for the species of origin, and was accepted as an ortholog if the top hit of this reciprocal blast was the same as the original protein. For statistical analyses of enrichment of ageing and immunity genes among queen biased genes, we used a strict, conservative version of this method in which the reciprocal blast needed to be the exact same NCBI entry as the original protein. We also report annotations obtained by a looser method in which we manually checked the reciprocal blast to determine

whether it represented a different isoform of the same original protein. These annotations are listed in Supplementary Tables S2 and S3.

To determine whether queen- or worker-biased genes were enriched for ageing genes, we counted the number of significantly queen-biased or worker-biased *L. niger* genes that were either annotated as an ageing gene or not annotated as an ageing gene, and applied a Fisher's exact test on the resulting 2x2 contingency table. The same procedure was used to determined whether queen- or worker-biased genes were enriched for immunity genes.

To determine whether differentially-expressed ageing genes were associated with increased or decreased longevity, we used the information present in the *GenAge* database, where genes are classed as “pro-longevity” or “anti-longevity” depending on whether expression is believed to increase or decrease lifespan. We compared the number of pro- or anti-longevity genes that were queen-biased or worker-biased using a Fisher's exact test. *L. niger* genes that were homologous to more than one *GenAge* entry with conflicting putative longevity effects were excluded from this analysis.

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Authors Contributions

ERL and LK conceived and designed the experiments and wrote the manuscript; ERL performed the experiments and analysed the data.

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Table 1: Number of ageing genes vs non-ageing genes and immunity genes vs non-immunity genes that are significantly (FDR < 0.05) differentially expressed between queens and workers in legs and brains of 1-day-old and 2-month-old individuals. Q-biased = queen-biased; W-biased = worker-biased; 1d = 1-day-old; 2m = 2-months-old. Each 2x2 sub-table (within an age / tissue context for either ageing or immunity genes) is a contingency table on which a Fisher's Exact test can be performed. Non-ageing and non-immunity genes are defined as genes with no homology to genes in the ageing or immunity databases respectively.

	Q-biased 1d legs	W-biased 1d legs	Q-biased 2m legs	W-biased 2m legs	Q-biased 1d brains	W-biased 1d brains	Q-biased 2m brains	W-biased 2m brains
Ageing genes	96	34	71	93	17	4	4	0
Non-ageing genes	2555	3107	4190	6046	992	371	352	130
Immunity genes	23	7	18	18	11	1	5	2
Non-immunity genes	2628	3134	4243	6121	998	374	351	128

Table 2: Number of pro-longevity and anti-longevits genes, as classified in the GeneAge database, that are significantly (FDR < 0.05) differentially-expressed between queens and workers in legs and brains of 1-day old and 2-months old individuals. Q-biased = queen-biased; W-biased = worker-biased; 1d = 1-day-old; 2m = 2-months-old. Each 2x2 sub-table (within an age / tissue context) is a contingency table on which a Fisher's Exact test can be performed.

	Q-biased 1d legs	W-biased 1d legs	Q-biased 2m legs	W-biased 2m legs	Q-biased 1d brains	W-biased 1d brains	Q-biased 2m brains	W-biased 2m brains
Pro-longevity	35	9	28	22	9	2	2	0
Anti-longevity	58	24	37	64	6	1	1	0

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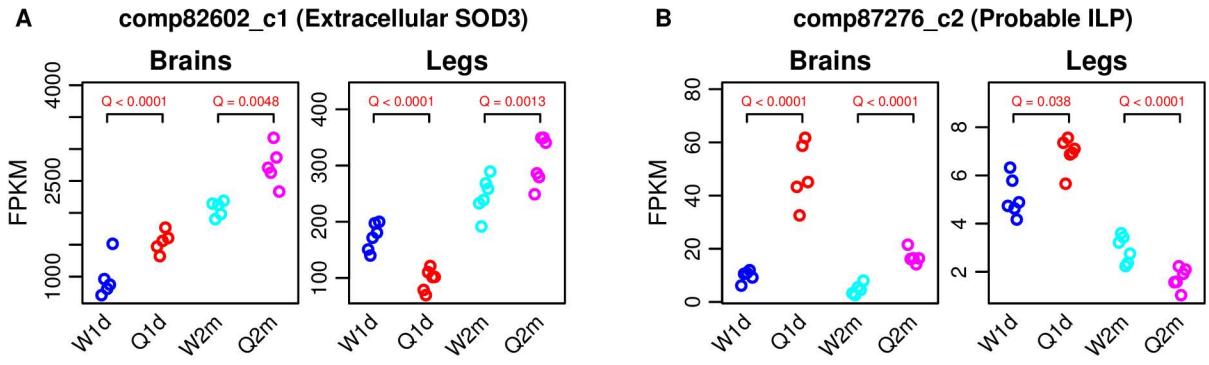

Figure 1: Expression level (Fragments Per Kilo-base per Million reads) of two of the most significantly differentially expressed genes between queens and workers. Points are jittered on the x axis to show overlapping data. W1d: 1-day-old workers; Q1d: 1-day-old queens, W2m: 2-month-old workers; Q2m: 2-month-old queens.