Genotype effect on lifespan following *vitellogenin* knockdown

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Abstract

Honey bee workers display remarkable flexibility in the aging process. This plasticity is closely tied to behavioral maturation. Workers who initiate foraging behavior at earlier ages have shorter lifespans, and much of the variation in total lifespan can be explained by differences in pre-foraging lifespan. Vitellogenin (Vg), a yolk precursor protein, influences worker lifespan both as a regulator of behavioral maturation and through anti-oxidant and immune functions. Experimental reduction of *Vg* mRNA, and thus Vg protein levels, in wild-type bees results in precocious foraging behavior, decreased lifespan, and increased susceptibility to oxidative damage. We sought to separate the effects of Vg on lifespan due to behavioral maturation from those due to immune and antioxidant function using two selected strains of honey bees that differ in their phenotypic responsiveness to *Vg* gene knockdown. Surprisingly, we found that lifespans lengthen in the strain described as behaviorally and hormonally insensitive to *Vg* reduction. We then performed targeted gene expression analyses on genes hypothesized to mediate aging and lifespan: the *insulin-like peptides* (*Ilp1* and *2*) and *manganese superoxide dismutase (mnSOD*). The two honey bee *Ilps* are the most upstream components in the insulin-signaling pathway, which influences lifespan in *Drosophila melanogaster* and other organisms., while *manganese superoxide dismutase* encodes an enzyme with antioxidant functions in animals. We found expression differences in the *Ilps* in fat body related to behavior (*Ilp1 and 2)* and genetic background (*Ilp2)*, but did not find strain by treatment effects. Expression of *mnSOD* was also affected by behavior and genetic background. Additionally, we observed a differential response to *Vg* knockdown in fat body expression of *mnSOD,* suggesting that antioxidant pathways may partially explain the strain-specific lifespan responses to *Vg* knockdown.

Keywords: vitellogenin, RNA-interference, *Apis mellifera,* foraging, longevity

Abbreviations: vitellogenin (Vg), insulin/insulin-like signaling (IIS), insulin-like peptide 1 (Ilp1), insulin-like peptide 2 (Ilp2), manganese superoxide dismutase (mnSOD), RNA-interference (RNAi), juvenile hormone (JH), double-stranded RNA (dsRNA), injected control treatment (injC), non-injected control treatment (noREF), *vitellogenin* knockdown treatment (*vg*RNAi)

1. Introduction

The extraordinarily plastic nature of honey bee life history and aging has recently made this eusocial insect a model organism for the study of senescence (Amdam et al., 2005; Remolina et al., 2007; Corona et al., 2007). From a single genome, female honey bees can develop along three very different aging trajectories triggered by environmental conditions: queens, which typically live between 1-2 years (Page and Peng, 2001); spring and summer workers, which live between 15-60 days (Fukuda and Sekiguchi, 1966); and diutinus workers (winter bees), which emerge in the late summer and early autumn in temperate regions and can live 6-8 months (Maurizio, 1950). Additionally, worker aging is not entirely chronological. Instead, senescence in workers appears to also be closely tied to behavioral maturation (Neukirch, 1982; Seehuus et al., 2006a; Behrends et al., 2007; Rueppell et al., 2007; Baker et al., 2012).

Honey bee workers exhibit an age-associated division of labor in which young (nurse) bees perform in-hive tasks such as brood care and food storage while older bees leave the nest to forage (Winston, 1987). The age at which a worker initiates foraging is strongly correlated with lifespan: the earlier a worker leaves the nest to forage, the shorter her lifespan is likely to be (Neukirch, 1982; Robinson et al., 1992a: Rueppell et al., 2007). This age-related behavioral progression can be slowed, accelerated, or even reversed based on internal, social, and environment factors (Robinson et al., 1992b; Huang et al., 1998; Pankiw and Page, 2001). The dynamic nature of the aging process in honey bees has enabled targeted studies on the physiological and molecular pathways involved in their aging and senescence (Münch and Amdam, 2010; Amdam, 2011).

Aging and lifespan in honey bees are affected in several ways by Vitellogenin (Vg), a yolk precursor protein. Vg is the most abundant protein in the hemolymph of nurse bees, comprising 30-50% of total protein (Engels and Fahrenhorst, 1974), and impacts both anti-oxidant and immune function (Amdam et al., 2004; Seehuus et al., 2006b; Corona et al., 2007). In honey bees, Vg appears to be the primary zinc carrier, and hemolymph zinc levels are closely

tied to fluctuations in Vg titer (Amdam et al., 2004). In foragers, a behavioral stage with low Vg titers, zinc levels fall so low that apoptosis is induced in hemocytes, cells that function in the innate immune response of insects (Amdam et al., 2005). Additionally, Vg itself has anti-oxidant properties and is preferentially carbonylated in response to oxidative damage induced via paraquot injection (Seehuus et al., 2006b). When faced with an oxidative challenge, caged workers with experimentally reduced *Vg* expression have higher mortality than control workers (Seehuus et al., 2006b).

Vg also impacts honey bee lifespan and aging by mediating the pacing of behavioral maturation in concert with juvenile hormone (JH). In insects JH is typically a gonadotropin associated with high titers of Vg (Flanagan and Hagedorn, 1977; Chen et al., 1979; Flatt et al., 2005). In honey bees, however, Vg and JH function in a mutually repressive feedback loop (Ramamurty and Engles, 1977; Pinto et al., 2000; Guidugli et al., 2005). Nurse bees have high Vg titers and low JH titers, while foragers have low Vg titers and high JH levels (Rutz and Lüscher, 1974; Rutz et al., 1976; Engels and Fahrenhorst, 1974; Robinson et al., 1991). *Vg* knockdown in the fat body induces early onset of foraging behavior and decreased JH titers (Guidugli et al., 2005; Nelson et al., 2007; Marco Antonio et al., 2008). Likewise, treatment with JH and its analogues induces early foraging and decreased Vg titers (Ramamurty and Engles, 1977; Robinson, 1987; Robinson et al., 1992a). In free-flying workers, *Vg* knockdown in the fat body decreases lifespan, likely through a combination effects on behavior, immunity and oxidative stress resistance (Nelson et al., 2007).

Studies using two selected lines of honey bees demonstrated that the measured physiological and behavioral responses to *Vg* knockdown are genotype dependent (Amdam et al., 2007; Ihle et al., 2010). The high and low pollen hoarding strains were bidirectionally selected for colony levels of pollen stores, but also exhibit a variety of differences at the level of behavior, physiology, and gene expression (reviewed in Page and Fondrk, 1995; Page et al., 2012; Page 2013). The sensitivity of the Vg/JH feedback loop is one such trait that differs between the strains (Amdam et al., 2007; Ihle et al., 2010). High

strain bees have higher peak titers of Vg that decline faster at the onset of foraging, relative to low strain bees (Amdam et al., 2007). Experimental manipulations suggest that this could be due to a stronger coupling of the Vg/JH feedback relationship in the high strain: in response to *Vg* knockdown, high strain bees exhibit an increase in JH titers while the low strain does not (Amdam et al., 2007). High strain workers, like wild-type, forage earlier in response to *Vg* knockdown in the fat body, while behavioral maturation was not affected by *Vg* knockdown in the low strain (Ihle et al., 2010).

We investigated how *Vg* influences lifespan in the genetic background of the high and low strains to separate its effects on lifespan due to behavioral maturation from those due to antioxidant and immunological functions. In the Vg-responsive high strain, we predicted that *Vg* knockdown would decrease lifespan consistent with results in wild-type bees. In the low strain, where *Vg* knockdown does not appear to affect behavioral maturation and JH dynamics, we predicted either no response or a decreased lifespan reflecting reduced protection from oxidative and immune challenges.

Lifespan of high strain workers was not significantly affected by *Vg* knockdown. Contrary to our prediction, *Vg* knockdown increased lifespan in the low strain. We hypothesized that the observed genotype-specific differences in lifespan after *Vg* knockdown could be traced to differentially effective compensatory mechanisms between the strains. We then performed targeted expression analysis on same age nurses and foragers of both strains to investigate pathways associated with lifespan and oxidative damage.

The conserved insulin/insulin-like signaling (IIS) pathway is known to influence many important biological processes including aging, reproduction, and nutrition in both insects and vertebrates, (reviewed in Wu and Brown, 2006). The functions of the IIS pathway in honey bees are less well understood, but it has been shown to be associated with behavior (Ament et al., 2008; Wang et al., 2010), nutritional status (Ament et al., 2011; Nilsen et al., 2011; Ihle et al., 2014), and lifespan (Corona et al., 2007). Interestingly, the IIS pathway appears to be a central regulator of the divergent phenotypes of the high and low strains (Page et

al., 2012).

The most upstream components of the IIS pathway in honey bees, insulin-like peptides *(Ilp1* and 2), in particular have been liked to Vg and JH levels (Corona et al., 2007; Nilsen et al., 2011). Peripheral *Ilp1* expression is positively correlated with *Vg* expression (Nilsen et al., 2011), while expression of *Ilp1* in the head is increased by JH-analogue treatment (Corona et al., 2007). *Ilp1* expression increases in response to sugar and amino acid supplementation (Ament et al., 2011; Nilsen et al., 2011; Ihle et al., 2014) and has been implicated in the extended lifespan phenotype of queens (Corona et al., 2007). In contrast, *Ilp2* expression is correlated with JH titer (Nilsen et al., 2011), and does not respond to nutrient manipulation (Ament et al., 2011; Nilsen et al., 2011; Ihle et al., 2014). Expression of both peptides appears to be sensitive to behavioral maturation or age (Corona et al., 2007; Ament et al., 2011). Thus, we hypothesized that *Ilp1* and *Ilp2* could mediate the divergent lifespan responses to *Vg* knockdown observed in the high and low strains.

As Vg may affect lifespan by mitigating oxidative damage, we further reasoned that low strain workers may have compensatory mechanisms that shield them from some of the potential deleterious effects of naturally low *Vg* expression, which may be particularly sensitive to *Vg* knockdown. We targeted *manganese superoxide dismutase* (mnSOD), because as a mitochondrial antioxidant important during aerobic respiration, its activity may be especially key during the aerobically challenging flight of honey bee foragers (Fridovich, 1995). Expression dynamics of *mnSOD* differ between queens and workers and can be associated with aging (Corona et al., 2005).

Bidirectional selection has demonstrated that some honey bees can respond to reduced Vg levels with increased lifespan, perhaps via alternative mechanisms of self-maintenance. This low strain phenotype is associated with severely reduced Vg sensitivity in behavioral and hormonal regulation, and may be uncommon in wild type that relies on intact Vg functions.

2. Materials and Methods

2.1. Bees

Worker progeny were derived from queens in the 33rd generation of selection. High pollen strain colonies stored nearly 7 times the amount of low pollen strain colonies. Mean area of pollen for highs (n=16) was 173.2 square inches and the mean for low colonies (n=17) was 24.8 square inches (Mann-Whitney: U=2, *Z*=4.74, p>0.0001). Queens from two source high and low strain colonies were caged over night to allow easy collection of same-aged bees for both the lifespan and gene expression trails. After 20 days, frames were removed from the colonies and worker bees were emerged in an incubator set at 34°C with approximately 70% relative humidity. Newly emerged bees were randomly assigned to one of three groups: *vg*RNAi, the experimental dsRNA injected group, the double control groups of noREF, a non-handled reference group; and injC, a control group injected with vehicle, according to established protocols, e.g. (Guidugli et al., 2005; Nelson et al., 2007). Bees to be used in the longevity experiment (below) were individually tagged (BeeWorks, Orillia, Ontario, Canada) while bees used in the gene expression study were marked with paint (Testors Enamel; Testor Corporation, Rockford, Illinois, United States) to indicate treatment group.

2.2. dsRNA preparation and injection

Double stranded RNA (dsRNA) toward *Vg* was prepared according to the RNA interference (RNAi) protocol of Amdam et al. (Amdam et al., 2003; Amdam et al., 2006). The cDNA clone AP4a5 was used as template (GenBank accession #: AJ517411), and primers were fused to T7 promoter sequence (underlined): Fw: 5'-TAATACGACTCACTATAGGGCGAACGACTCGACCAACGACTT-3', Re: 5'-TAATACGACTCACTATAGGGCGAAACGAAAGGAACGGTCAATTCC-3'. PCR product was purified using the QIAquick PCR purification kit (Qiagen, Valencia, California, United States), and RNA was prepared with the Promega RiboMax T7 system (Promega, Madison, Wisconsin, United States). RNA was extracted by

TRIzol LS reagent (GIBCO-BRL, San Diego, California, United States), resuspended in nuclease-free water, heated at 96°C for 2 min, and left to cool at room temperature for 20 min. dsRNA products were brought to a final concentration of 5 µg/µl in nuclease-free water (Qiagen) (Amdam et al., 2003).

Before injection bees were cold anaesthetized, and secured to wax covered plates by crossing pins between the thorax and abdomen. Injections were performed between the fifth and sixth tergites using Hamilton syringes with G30 disposable needles (BD, Palo Alto, California, United States). Injection volume was 2 µl for both *vg*RNAi and injC groups.

2.3. Lifespan

Injections took place over three days for each colony. Treated bees (n = 200 bees per treatment, per genotype, per colony) were individually tagged, and placed into each of two, three-framed, glass-walled observation colonies with a wild-type background population. Colonies were surveyed in the evening after all foragers had returned for the night. Each frame was scanned twice per side, and all ID tags were recorded to determine surviving individuals. Age of death was considered to be the day after the last sighting of an individual (Nelson et al., 2007).

We determined which bees had initiated foraging during our study window by monitoring glass-topped runways into the colonies during peak foraging windows. The ID tags of all returning foragers were recorded. Individuals observed returning from foraging trips more than once were considered confirmed foragers in our analyses. We determined total lifespan for the experimental population as a whole as well as for confirmed foragers. Additionally, we divided total lifespan into pre- and post-foraging initiation components. Pre-foraging lifespan was determined by age of foraging initiation or age of death if death occurred before the onset of foraging behaviour. Post-foraging lifespan was considered to be the span between foraging initiation and death.

2.4. Gene expression

Injections took place on a single day for each colony. Treated bees (n = 100 bees per treatment, per genotype, per colony) were paint marked to indicate treatment group (*Vg*RNAi or injC) and introduced into one of two five-frame nuclear colonies with a wild-type background population. Treated bees were allowed to mature inside the host colonies for 10 days. 10-day old bees were collected from the colonies at 8:00am to avoid the circadian fluctuations of antioxidant gene expression observed previously (Williams et al., 2008; Elekonich, 2009) and to enable collection of both nurses and foragers. Bees identified as foragers were collected as they alighted on the colony entrance after returning from a flight. Nurses were identified as bees that had been observed to put their heads into cells containing young brood. Fat body and brain were dissected as described before (Nelson et al., 2007; Nunes et al., 2013). Briefly, fat body tissue was harvested by removing the gut tract, leaving fat body tissue clinging to the abdominal carcase.

RNA was extracted from fat body tissue using a combined Trizol (Invitrogen) and RNeasy Kit (Qiagen) method according (Amdam et al., 2003). Brain RNA was obtained through phenol:chloroform extraction. We measured RNA quality and concentration using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and diluted all samples to 25 µg/µL. Relative gene expression levels were determined by one-step reverse transcription- polymerase chain reaction (RT-qPCR) using QuantiTect SYBR Green RT-PCR Master Mix kit (Qiagen) and ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA). All samples were run in triplicate. Negative controls, samples run without the addition of the RT enzyme, confirmed the absence of genomic DNA contamination. Knockdown of *Vg* in fat body was verified relative to injected controls as previous work has demonstrated that injected controls do not differ from the non-handled reference group (Amdam et al., 2007; Nelson et al., 2007). Transcript levels of *Vg* (accession # AJ517411), *Ilp1* (GB17332-PA), *Ilp2* (accession # GB10174_PA) and *mnSOD* (accession # AY329356) were quantified relative to *β-actin* (accession # AB023025)

285 expression using real-time RT PCR as before e.g. (Amdam et al., 2004;
286 Lourenco et al., 2008). β-*actin* is stably expressed in several tested honey bee
287 tissues and has been demonstrated to be an effective control gene when
288 measuring gene expression in adult honey bee fat body (Lourenco et al., 2008;
289 Scharlaken et al., 2008). As such, β-*actin* is a commonly used reference in
290 studies of honey bee gene expression (Chen et al., 2005). Primers are listed in
291 Supplementary Table 1. Control reactions without reverse transcriptase were
292 preformed for each sample to ensure reactions were not contaminated with
293 genomic DNA.
294
295 *2.5. Statistical analysis*
296 Lifespan data were analyzed using Kaplan-Meier survival analysis (Amdam et al.
297 2007). Planned pair-wise comparisons were made between the vgRNAi and injC
298 groups as well as between the injC and noREF groups with the Cox-Mantel test
299 (Nelson et al., 2007; Ihle et al., 2010). Gene expression data were log
300 transformed to approximate a normal distribution, and analyzed by factorial
301 ANOVA (Rieu and Powers, 2009; Wang et al., 2010). Post-hoc analysis was
302 performed with Fisher's LSD test. All analyses were conducted using Statistica
303 6.0 (StatSoft, Inc. Tulsa, Oklahoma, United States).
304
305 **3. Results**
306
307 *3.1. Knockdown verification*
308 We first confirmed *Vg* knockdown in fat body ($F_{(1, 79)}$ =67.20, $p < 0.0001$). *Vg*
309 expression was significantly reduced by *Vg* dsRNA injection across strains and
310 task group (LSD: high strain nurses $p < 0.0001$, high strain foragers $p < 0.0001$,
311 low strain nurses $p = 0.0020$, low strain foragers $p=0.0008$; df = 80; Fig. 3A-D).
312
313 *3.2. Lifespan effects of* Vg *knockdown*
314 We tested the effects of *Vg* knockdown on the lifespan of all experimental bees
315 as well as on the lifespan of confirmed foragers. When we considered the entire

316 experimental population, we found that in contrast to results from wild type
317 (unselected) bees (Nelson et al., 2007), *Vg* knockdown had no effect on the
318 lifespan of the high strain bees (Kaplan-Meier χ^2 = 3.517, df = 2, p = 0.1723; n=
319 noREF:189, injC:227, and VgRNAi:211; Fig. 1A). There was a significant effect of
320 treatment in the low strain (Kaplan-Meier χ^2 = 30.576, df = 2, p < 0.0001; n=
321 noREF:233, injC:210, and VgRNAi:212; Fig. 1B). Intriguingly, *Vg* knockdown in
322 the low strain increased lifespan relative to injected controls (Cox-Mantel U =
323 25.4983, p = 0.0067).
324 Within the confirmed forager population, there was an overall effect of
325 treatment in the high strain (Kaplan-Meier χ^2 = 7.2342, df = 2, p = 0.0268; n =
326 noREF: 113, injC:173, and VgRNAi:169). The differences between treatment
327 groups to be compared were not significant (Cox-Mantel: inC vs. VgRNAi U =
328 11.066, p = 0.173; injC vs noREF U = -7.259, p = 0.310), but we observed a non-
329 significant pattern mirroring that seen in wild-type bees with vgRNAi bees dying
330 earlier than controls (Nelson et al., 2007). There was also an effect of treatment
331 in low strain confirmed foragers (Kaplan-Meier χ^2 = 17.7264, df = 2, p < 0.0001;
332 n= noREF:128, injC:91, and VgRNAi:107). *Vg* knockdown resulted in increased
333 lifespan in confirmed low strain foragers relative to the control (Cox-Mantel: injC
334 vs. VgRNAi U = -16.92, p = . 0.0052). Additionally, there was a significant
335 negative effect of handling on lifespan (Cox-Mantel: injC vs noREF U = -23.02, p
336 < 0.0001).
337 While *Vg* knockdown did not affect total lifespan in the high strain, it did
338 affect both the pre-foraging (Kaplan-Meier χ^2 = 13.898, df = 2, p < 0.001) and
339 post-foraging initiation (Kaplan-Meier χ^2 = 7.323, df = 2, p = 0.0270) lifespans,
340 albeit in different directions. In the high strain, *Vg* knockdowns had significantly
341 shorter pre-foraging lifespans than did injected controls (Cox-Mantel: injC vs.
342 vgRNAi U = -28.596, p < 0.01; Fig 2A) consistent with their early foraging
343 initiation (Ihle et al., 2010). Likewise, high strain vgRNAi bees had significantly
344 longer post-foraging onset lifespans than the injC group (Cox-Mantel: inC vs.
345 VgRNAi U = 13.899, p = 0.0482; Fig 2B), displaying an established negative
346 correlation between pre- and post-foraging initiation components of lifespan

(Neukirch, 1982; Rueppell et al., 2007). In the low strain, there was no effect of treatment on either pre-foraging lifespan (Kaplan-Meier $\chi^2 = 22.878$, df = 2, $p < 0.0001$; Fig. 2C) or post-foraging initiation lifespan (Kaplan-Meier $\chi^2 = 0.424$, df = 2, $p = 0.8090$; Fig 2D), suggesting that the increase in overall lifespan is not an artifact of the age of foraging, a trait unaffected by *Vg* reduction in the low strain.

3.2. Gene expression

Vg expression in fat body was significantly affected by strain ($F_{(1, 79)} = 11.99$, $p = 0.0009$), treatment ($F_{(1, 79)} = 67.20$, $p < 0.0001$) and task ($F_{(1, 79)} = 53.39$, $p < 0.0001$), but not colony ($F_{(1, 79)} = 2.08$, $p > 0.152$). Fat body *Vg* expression was not different between high and low strain injected control (LSD: $p = 0.8670$, df = 78) or injected control foragers (LSD: $p = 0.0886$, df = 78). A significant strain x treatment effect ($F_{(1, 79)} = 5.85$, $p = 0.0178$) indicates that the knockdown was stronger in high strain bees. *Vg* expression in brain was upregulated in response to knockdown in abdominal fat body (Fig 3E-H). Expression of *Vg* in brain was affected by treatment ($F_{(1, 73)} = 16.2737$, $p < 0.0001$) and task ($F_{(1, 73)} = 8.1764$, $p = 0.0055$), but not by strain ($F_{(1, 73)} = 1.3415$, $p = 0.25$) or colony ($F_{(1, 73)} = 3.5260$, $p = 0.0643$). Expression of *Vg* in brain is higher in foragers than it is in nurses (LSD: $p = 0.0006$, df = 74), and higher in the vgRNAi group than in injected controls (LSD: $p < 0.0001$, df = 74).

Ilp1 expression in the abdominal fat body was influenced by task (Factorial ANOVA: $F_{(1, 79)} = 28.93$, $p < 0.0001$), but not by strain ($F_{(1, 79)} = 0.126$, $p = 0.7240$), treatment ($F_{(1, 79)} = 3.03$, $P > 0.0854$), or colony ($F_{(1, 79)} = 1.75$, $P > 0.189$). When the strains were considered separately, the effect of task was independently significant with nurses having higher expression than foragers in both high and low strains (LSD: high strain $p < 0.0001$; low strain $p < 0.0013$). There was no influence of strain ($F_{(1, 69)} = 0.62$, $p = 0.4354$), treatment ($F_{(1, 69)} = 1.28$, $p = 0.2620$), task ($F_{(1, 69)} = 0.63$, $p = 0.4290$), or colony ($F_{(1, 69)} = 0.49$, $p = 0.4840$) on *Ilp1* expression in brain.

As reported before, abdominal fat body expression of *Ilp2* was not affected by *Vg* knockdown (Factorial ANOVA: $F_{(1, 67)}=0.22$, $p = 0.7730$) (Nilsen et al.

2011). However, strain ($F_{(1, 67)}$= 18.75, $p < 0.0001$) and task ($F_{(1, 67)}$=11.82, $p < 0.001$) did influence *Ilp2* expression. The low strain has higher *Ilp2* expression than the high strain, (LSD: $p < 0.0001$, df = 68) and nurses have higher expression than foragers ($p < 0.0009$, df = 68). Brain *Ilp2* expression was not influenced by strain (Factorial ANOVA: $F_{(1, 78)} = 0.076$, $p = 0.783$), treatment ($F_{(1, 78)} = 0.86$, $p = 0.358$), or task ($F_{(1, 78)} = 2.47$, $p = 0.120$).

mnSOD expression in abdominal fat body was affected by strain (Factorial ANOVA: $F_{(1, 79)} = 6.81$, $p = 0.011$) and task ($F_{(1, 79)} = 16.85$, $p < 0.0001$), but not by colony ($F_{(1, 79)}$=1.23, $p = 0.271$) or treatment ($F_{(1, 79)} = 0.07$, $p = 0.7832$). Low strain bees had higher *mnSOD* expression than high strain bees (LSD: $p < 0.0034$, df = 80), and nurses had higher expression than foragers ($p < 0.0001$, df = 80). Additionally, there was a significant interaction effect between strain and treatment ($F_{(1, 79)} = 7.77$, $p = 0.0066$), revealing that the strains responded differently to *Vg* knockdown (Fig 6A-D). While the effects of treatment were not independently significant for either strain (LSD: high strain $p = 0.1150$, df = 80; low strain $p = 0.1470$, df = 80), there was a non-significant trend in the high strain for *Vg* knockdown to decrease *mnSOD* expression in abdominal fat body and a non-significant increase in the low strain. In brain tissue, *mnSOD* expression is influenced by task (Factorial ANOVA: $F_{(1, 78)} = 8.29$, $p = 0.0051$), but not strain ($F_{(1, 78)} = 1.48$, $p = 0.226$) or treatment ($F_{(1, 78)} = 1.20$, $p = 0.276$). In contrast to expression patterns in abdomen, foragers had higher expression *mnSOD* expression in head tissue than did nurses. However, when these results are broken down by strain, the effect was only significant in the low strain (LSD: low strain $p = 0.0122$; high strain $p = 0.607$, df = 80; Fig 6E-H).

4. Discussion

Using two divergently selected strains of honey bees that differ in the strength of their Vg/JH feedback relationships, we were able to uncover lifespan responses to *Vg* knockdown not observed in a previous study on wild-type bees (Nelson et al., 2007). In the total population of treated bees, lifespan was not significantly different between high strain *Vg* knockdowns and controls. Intriguingly, in the low

strain, in which *Vg* knockdown does not affect behavioral maturation, *Vg* knockdown resulted in increased lifespan relative to injected controls. We predicted that the lifespan effects of *Vg* knockdown would be more pronounced in the sub-population of confirmed foragers. Foraging honey bees have among the highest metabolic rates measured (Harrison et al., 1996). Therefore, foraging represents a major metabolic and oxidative challenge for worker bees. While foragers have lower hemolymph titers of Vg than nurses, RNAi against *Vg* further reduces expression levels in the fat bodies of both high and low strains, potentially exposing *Vg* knockdowns to increased oxidative damage (Seehuus et al. 2006). There was no significant effect of *Vg* knockdown in confirmed high strain foragers, but they did display a non-significant trend toward decreased lifespan in response to *Vg* knockdown, similar to results from wild-type (unselected) honey bees (Nelson et al., 2007). In the low strain, we again observed an increase in total lifespan in the vgRNAi group.

We initially hypothesized that the *Vg* knockdown-induced increase in lifespan observed in low strain workers could be the result of delayed foraging onset, as age of foraging initiation has been shown to be strongly correlated with lifespan (Neukirch, 1982; Robinson et al., 1992a; Rueppell et al., 2007). However, in the low strain we found no effect of *Vg* knockdown on either pre-foraging or post-foraging initiation lifespan. This suggests that the overall lifespan increases observed in low strain *Vg* knockdowns occur throughout both general life stages, but were not statistically significant within either component of total lifespan. In the high strain, *Vg* knockdowns initiated foraging earlier in life (Ihle et al., 2010) and had longer foraging lifespans than did controls, consistent with previous work demonstrating a strong negative relationship between pre-foraging and post-foraging onset lifespans (Rueppell et al., 2007).

As the increased lifespan in the low strain *Vg* knockdown group could not be explained by behavioral ontogeny, we hypothesized that it may instead be due to differential *Vg* response to knockdown or compensatory signaling from pathways that intersect *Vg* action and which are associated with aging and senescence. While RNAi-induced *Vg* knockdown in the fat body is effective in

both strains, the high strain experiences a greater reduction in *Vg* expression in the fat body than the low strain. This reflects the Vg dynamics in untreated low strain bees that naturally have slower declines in Vg titer than do high strain bees (Amdam et al., 2007). The slower Vg decline observed in the low strain is likely due to a weak Vg/JH relationship in this strain. We did not find the previously observed strain differences in *Vg* expression in the fat body (Amdam et al., 2007). However, at 10 days of age, the bees in this study were likely past peak expression, after which expression of *Vg* decreases faster in the high strain.

In brain, we found that *Vg* expression is upregulated in response to both natural (injC foragers relative to nurses) and experimental (vgRNAi nurses and foragers relative to injC nurses and foragers respectively) declines in fat body-produced Vg. Our results are in contrast to those of a recent study using wild-type bees which found that brain *Vg* expression decreased in response to injection of dsRNA against *Vg* into the hemolymph (Nunes et al. 2013). However, these bees were collected at 15 days of age while our sample came from 10 day old bees. It is possible that we observed a short-lived phenotype present only immediately after foraging initiation or that, despite care, different dissectors produced samples containing different tissue types. The mechanism by which *Vg* affects behavior is not yet clear, nor are the relative contributions of fat body and brain expression levels. Further study is needed to determine which cell types can express *Vg* in honey bees, and how fat body and brain expression of *Vg* are related.

We found no evidence that *Ilp1* or *Ilp2* expression mediates the strain-specific, *Vg* knockdown induced differences in lifespan. There were no strain or strain-by-treatment effects on *Ilp1* expression. In same-age nurses and foragers, we found that nurse bees have higher fat body *Ilp1* expression than do foragers. In caged bees, fat body *Ilp1* expression increases in response to available amino acids and carbohydrates demonstrating a nutrient signaling function for *Ilp1* independent of behavioral phenotype (Nilsen et al., 2011; Ihle et al., 2014) . Our results support these findings and extend them to free-flying bees as nurses have high nutritional stores relative to foragers (Crailsheim, 1986; Toth and

Robinson, 2005). We did not find previously reported differences in brain *Ilp1* expression correlated with task (Ament et al., 2008). However, as chronological age was not controlled in that study (Ament et al., 2008), the forager group was likely older than the nurse group. Thus, the increased brain expression of *Ilp1* observed in foragers may be due to a positive correlation between *Ilp1* expression and age (Corona et al., 2007).

We found that fat body *Ilp2* expression is higher in nurses than in foragers, a result consistent with the hypothesis that Ilp2 is a broad indicator of nutrient availability (Nilsen et al., 2011). Additionally, we found that fat body *Ilp2* expression is higher in low strain than in high strain workers. We have previously hypothesized that Ilp2 may be an antagonist of the honey bee insulin receptors and may suppress the metabolic changes that accompany the transition to foraging, but this has yet to be tested experimentally (Nilsen et al., 2011). Here, high expression of *Ilp2* in the fat body would act remotely to suppress transduction of the IIS pathway in brain tissue and so inhibit synthesis of JH, a downstream target. It is possible that the high expression of *Ilp2* in low strain bees plays a role in the reduced sensitivity of their Vg/JH relationship, and may slow the transition to forager physiology. While these findings do not support a role of *Ilp2* expression in the strain-specific lifespan response to *Vg* reduction, they do suggest that the low strain bees may be a valuable tool in determining the mechanisms that underlie not only the behavioral transition from nursing to foraging behavior but also the metabolic changes that accompany it.

Our data indicate that one potential mechanism underlying the longer lifespans in the low strain following *Vg* knockdown is an up regulation of genes associated with defense against oxidative damage. Low strain workers have higher fat body expression of *mnSOD*, which encodes an enzyme active in the degradation of superoxide radicals in mitochondria (Fridovich, 1995). Expression of *mnSOD* between the strains is differentially affected by *Vg* knockdown: expression is decreased slightly in the high strain and slightly increased in the low strain following *Vg* suppression. In low strain workers, brain expression of *mnSOD* is higher in foragers than in nurses. The generally higher *mnSOD*

expression in low strain bees may mean that they invest more heavily in alternative pathways to combat oxidative damage when Vg expression is generally low. As such, in response to *Vg* knockdown, they may be exposed to less oxidative damage than is the high strain. Perhaps in low strain workers, expression patterns of *mnSOD,* and potentially other genes that mitigate oxidative damage, combined with increased local expression of *Vg* in the brain buffer the effects of senescence generally observed in the brains of foragers enough to significantly lengthen lifespan in response to *Vg* knockdown. However, while oxidative damage has been shown to correlate with aging in many organisms (Bokov et al., 2004), more recent work has called into question a causal role for oxidative stress in aging (Perez et al., 2009; Salmon et al., 2010). Future work is needed to quantify how Vg titer impacts oxidative damage in the brains and peripheral tissues of free-flying honey bees, and whether such damage translates to functional declines.

In this study, we found evidence to suggest that bidirectional selection on behavior has altered systems of self-maintenance: In the relative absence of Vg, alternative mechanisms extend worker life in the low but not the high pollen hoarding strain. This response occurred in the physiological context of severely reduced Vg sensitivity and generally low Vg levels that characterize low strain workers throughout life (Amdam et al. 2004a, 2007). This phenotype is likely uncommon in wild type bees, which rely on intact Vg functions in regulation of health and behavior. However, the low strain developed in an artificial selective context that favored Vg deficiency because it reduced pollen hoarding. Vg deficiency has negative consequences for bee health (Amdam et al. 2004b; Seehuus et al. 2006b), but for each generation only the healthiest colonies were used in breeding of the high and low strains. The low strain colonies that were used in breeding may, therefore, have represented genotypes that were able to recruit alternative self-maintenance systems to replace Vg.

We were unable to demonstrate a clear molecular explanation for the extended lifespans in the low strain after *Vg* knockdown. However, we feel this

paper provide strong data to support the use of bidirectional selection in the study of honey bee health and aging.

5. Acknowledgements

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7. Figure Captions

Fig 1. *Vg* expression in (a-d) fat body and brain (e-h). Relative expression by strain, task and treatment group. (a-d) Fat body RNAi against *Vg* (vgRNAi) was highly effective in both strains and across behavioral groups, but the knockdown was stronger in the high strain. (e-h) In brain, *Vg* expression is higher in foragers than in nurses and higher in the *vg*RNAi group than in the injC group. Asterisks indicate p-values < 0.05.

Fig 2. Effect of *vitellogenin* knockdown on total lifespan in (a) high and (b) low strain bees. Panels display the cumulative proportion of treated bees still alive at a given point in time. There was no effect of treatment in high strain workers. In low strain workers the vgRNAi group, bees in which *Vg* was experimentally reduced from emergence, lived significantly longer than did the injC group, bees that received a control injection of the vehicle. Injection stress also impacted lifespan in the low strain with the noREF group, non-injected control bees, lived significantly longer than the injC treatment group.

Fig 3. Total lifespan divided into pre- and post-foraging initiation components for both high and low strains. (a) high strain vgRNAi bees have significantly shorter pre-foraging lives than do the injC bees consistent with their earlier foraging onset (Ihle et al., 2010). (b) In contrast, the post-foraging onset lifespans of high strain vgRNAi bees are significantly longer than those of injC bees. (c) In the low strain, while there was an overall effect of treatment, there was no difference in pre-foraging lifespan between the vgRNAi and injC groups. (d) There was no effect of treatment on the post-foraging initiation lifespan in the low strain.

Fig 4. *Ilp1* expression in (a-d) fat body and brain (e-h). Relative expression by strain, task and treatment group. (a-d) Fat body expression of *Ilp1* is higher in nurses than in foragers, but was not influenced by strain or treatment. (e-h)

Expression of *Ilp1* in brain was not affected by any of the factors included in this study.

Fig 5. *Ilp2* expression in (a-d) fat body and brain (e-h). Relative expression by strain, task and treatment group. (a-d) Fat body expression of *Ilp2* is higher in nurses than in foragers and higher in low strain workers than in high strain workers. *Vg* knockdown did not affect fat body expression of *Ilp2.* (e-h) Expression of *Ilp2* in brain was not affected by any of the factors included in this study.

Fig 6. *mnSOD* expression in (a-d) fat body and brain (e-h). Relative expression by strain, task and treatment group. (a-d) Fat body expression of *mnSOD* is higher in nurses than in foragers and higher in low strain workers than in high strain workers. *mnSOD* expression was not affected by *Vg* knockdown. However, there was a significant strain by treatment response revealing that the low strain vgRNAi group had increased *mnSOD* expression relative to the high strain vgRNAi group. (e-h). Brain expression of *mnSOD* is higher in foragers than in nurses, but this effect is driven by differences in the low strain.

Figure(s)

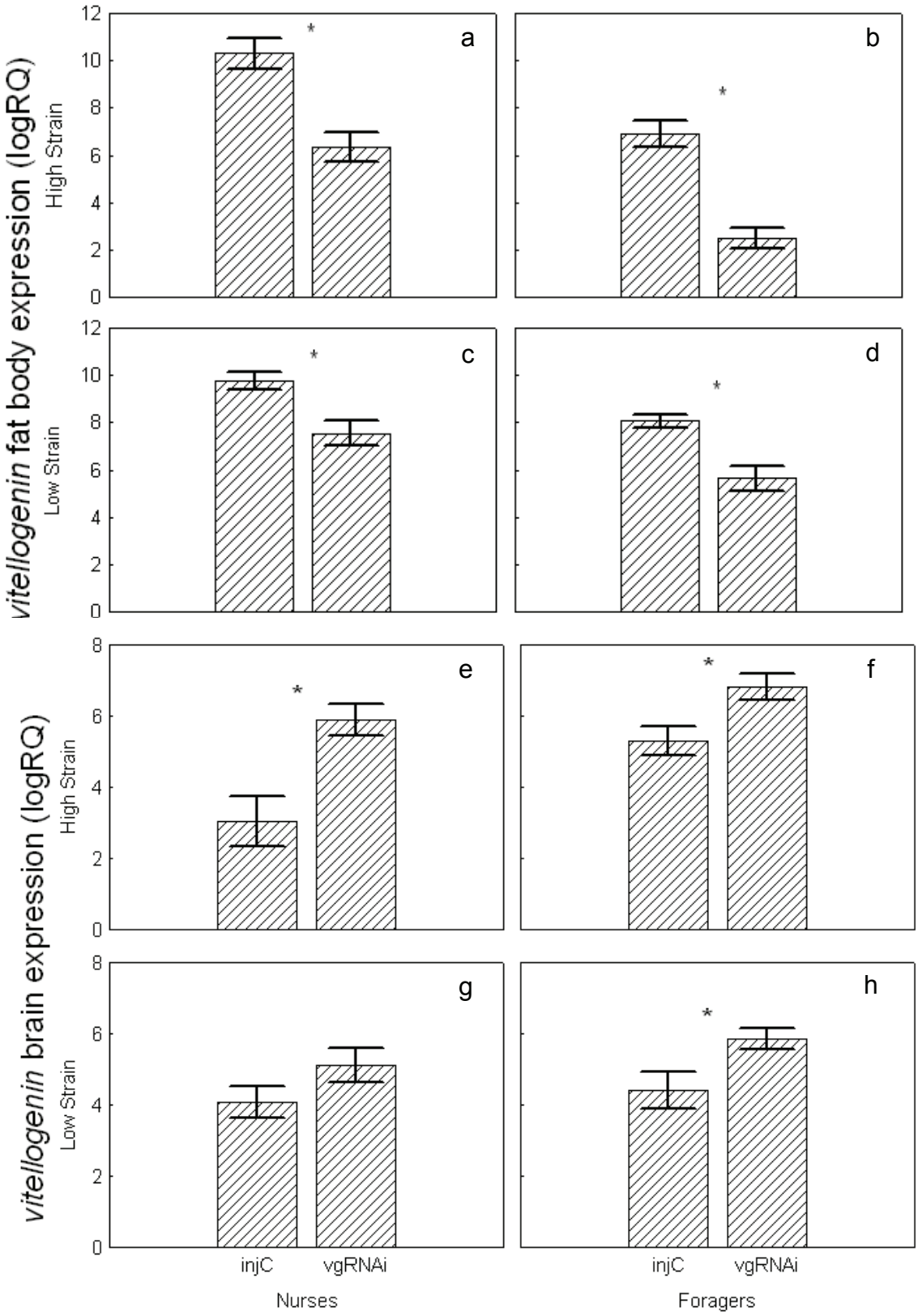

Figure 1

Figure(s)

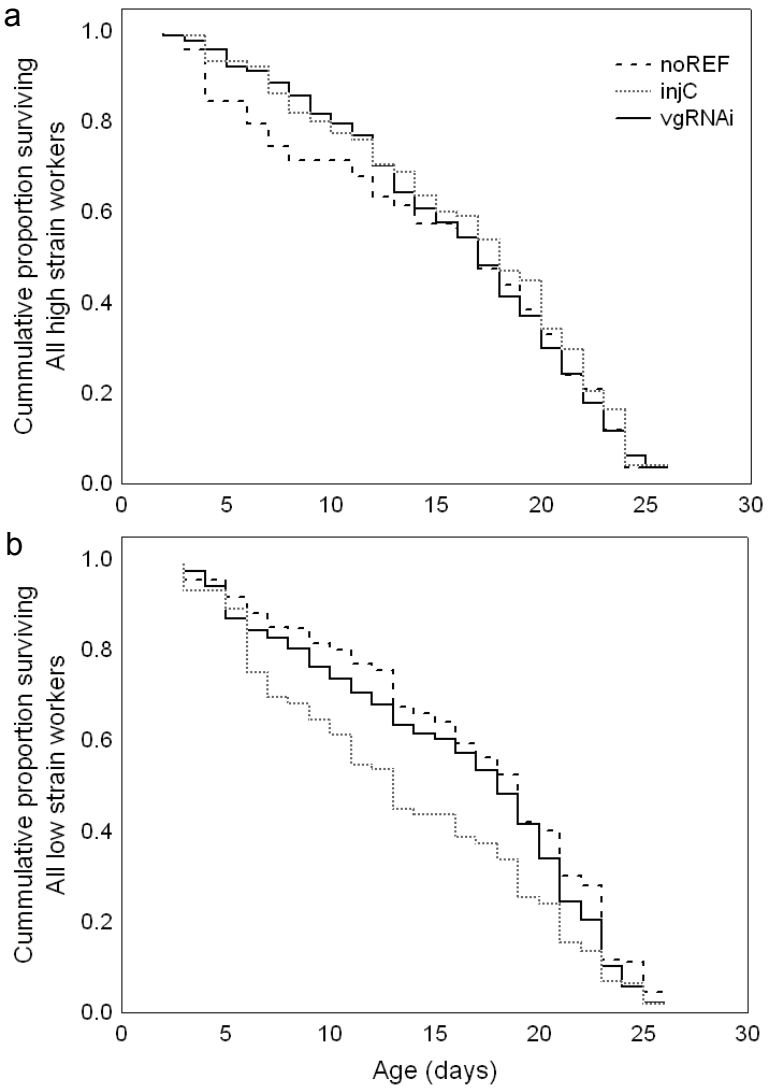

Figure 2

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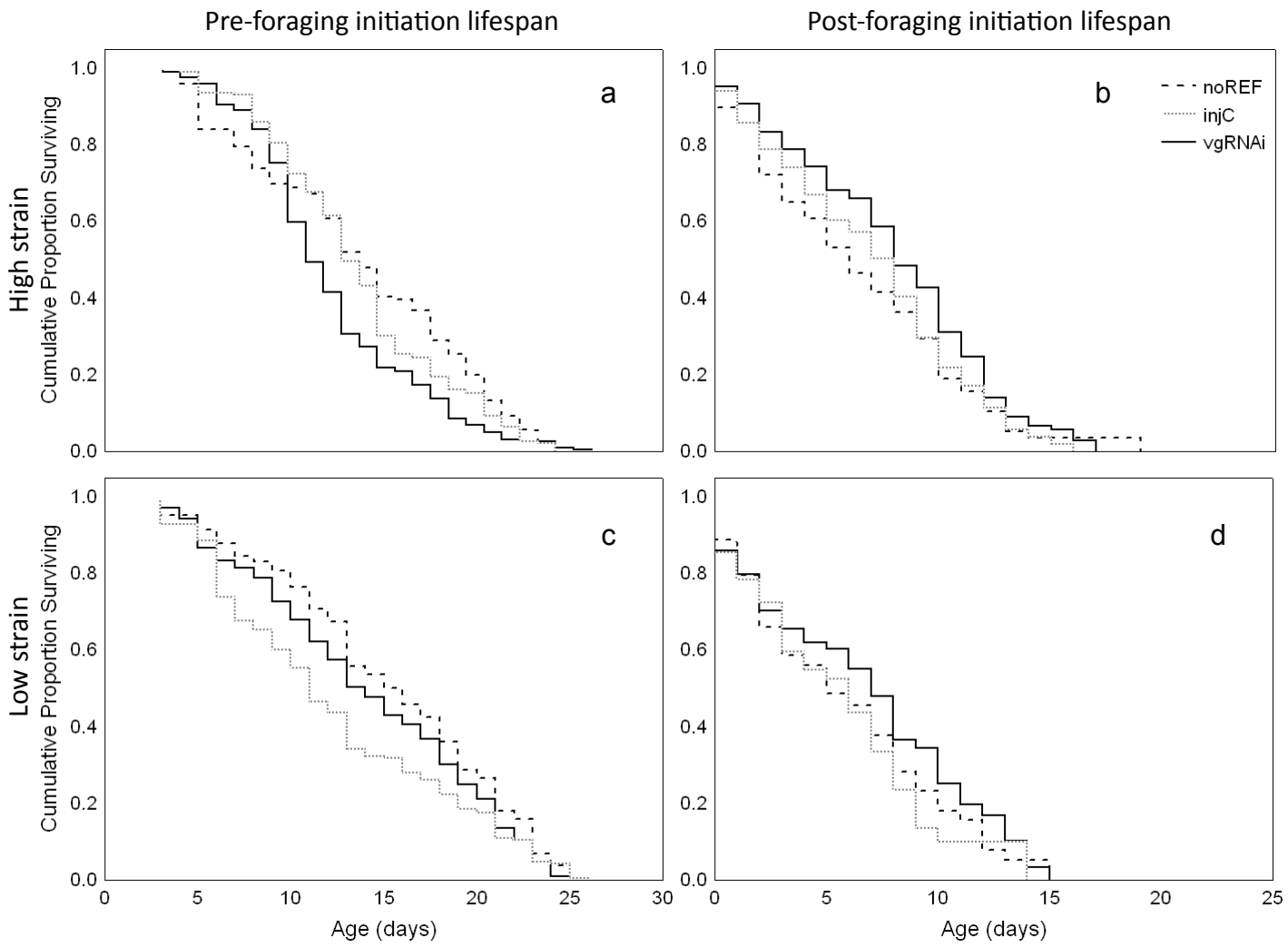

Figure 3

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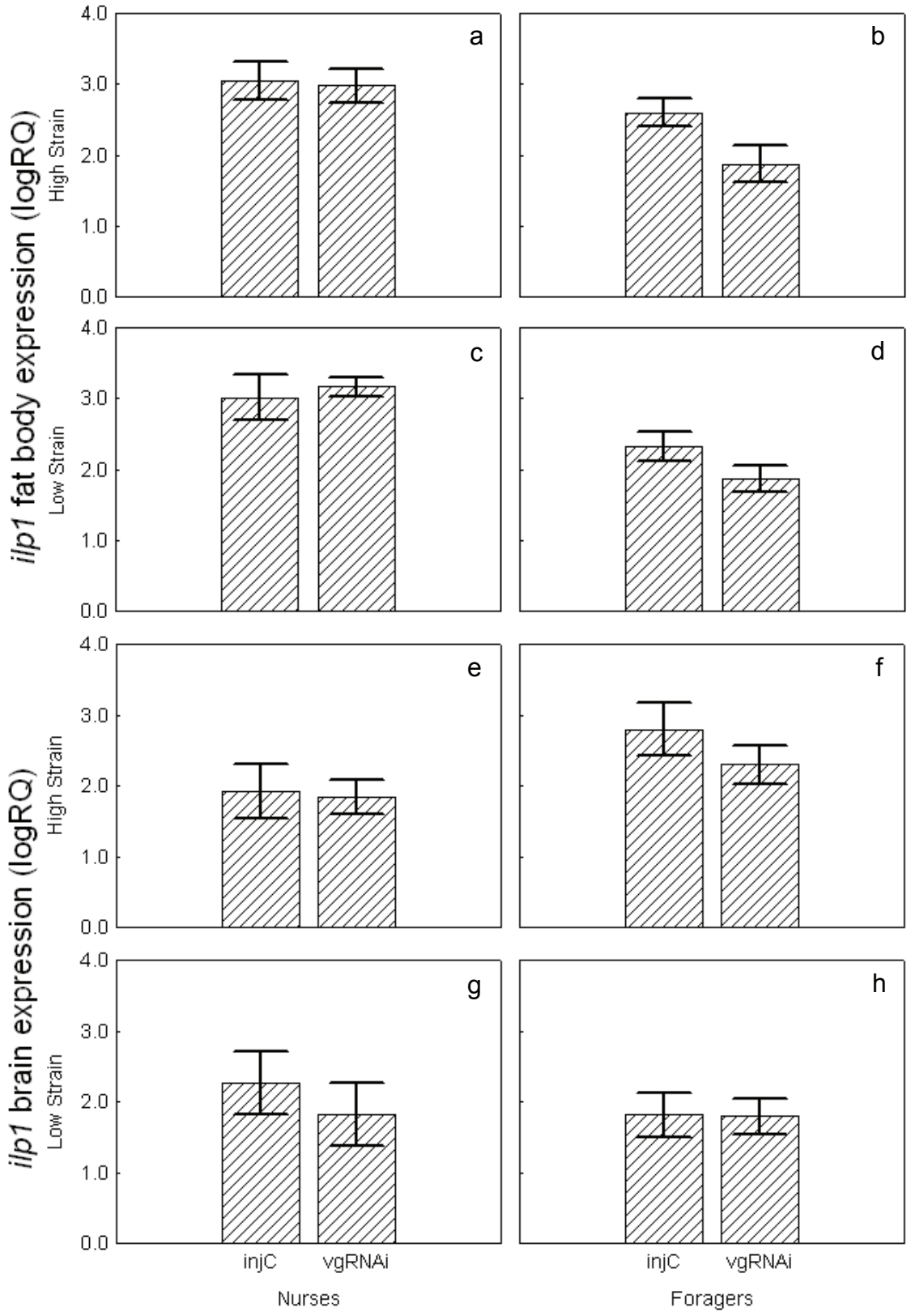

Figure 4

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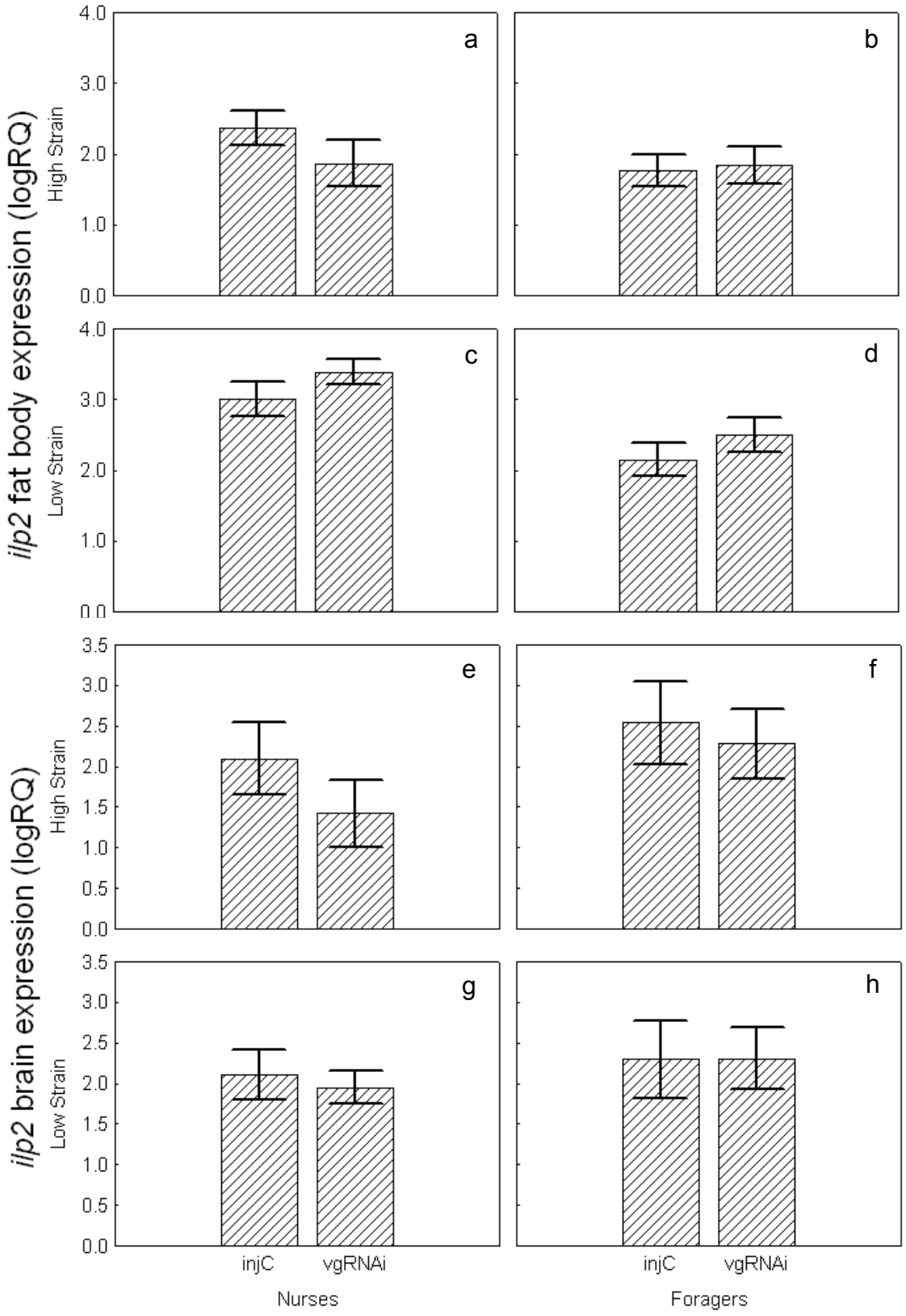

Figure 5

Figure(s)

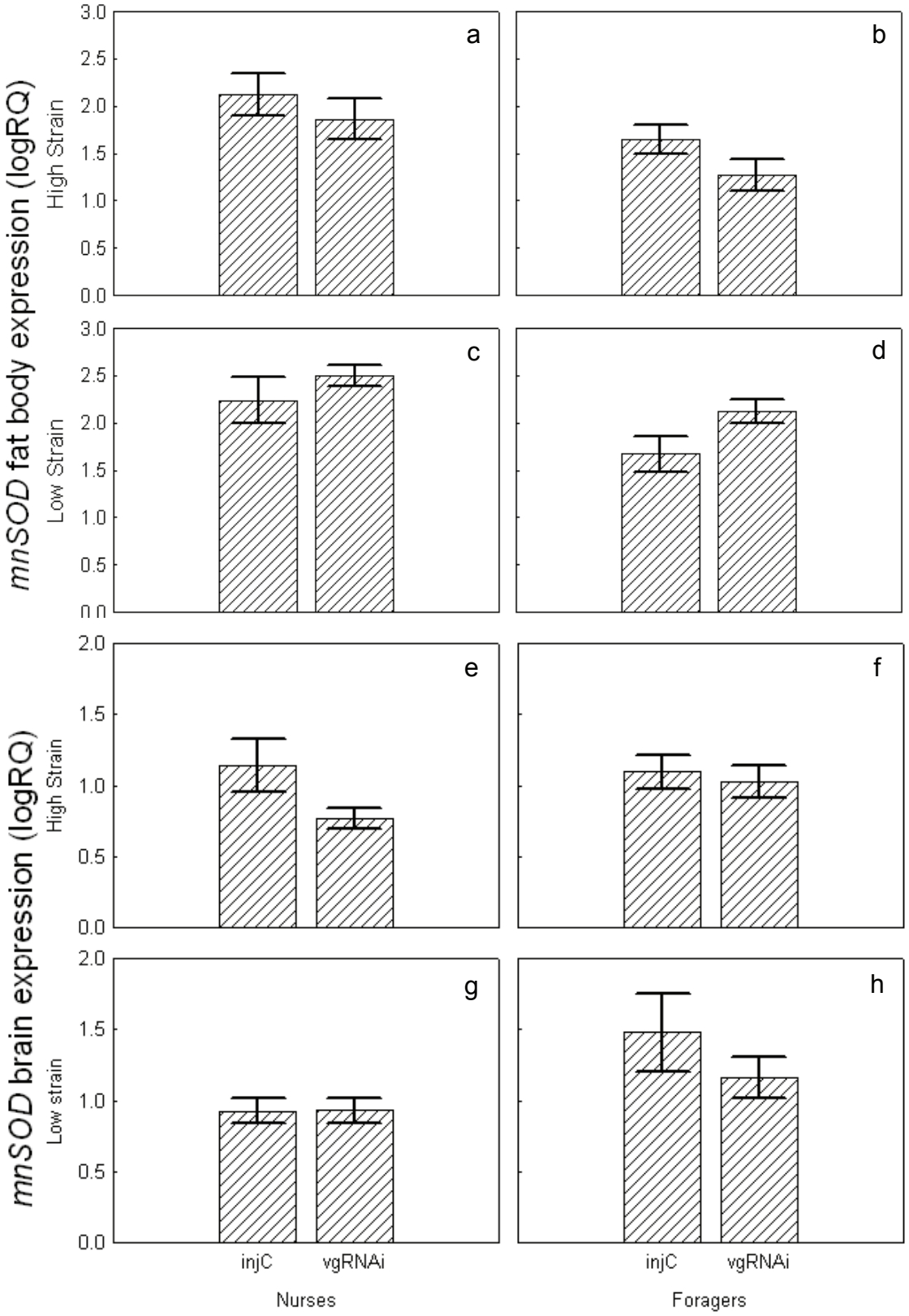

Figure 6

SUPPLEMENTAL INFORMATION

Table S1. List of primers and accession numbers.

Gene	Primer Sequences	Accession Number
β-actin	F: 5'-TGCCAACACTGTCCTTTCTG- 3' R: 5'-AGAATTGACCCACCAATCCA- 3'	AB023025
vitellogenin	F: 5'- GTTGGAGAGCAACATGCAGA - 3' R: 5'- TCGATCCATTCCTTGATGGT - 3'	AJ517411
insulin-like peptide 1	F: 5'-CGATAGTCCTGGTCGGTTTG- 3' R: 5'-CAAGCTGAGCATAGCTGCAC- 3'	GB17332-PA
insulin-like peptide 2	F: 5'- TTCCAGAAATGGAGATGGATG- 3' R: 5'-TAGGAGCGCAACTCCTCTGT- 3'	GB10174-PA
manganese superoxide dismutase	F: 5'- GGTGGTGGTCATTTGAATCATTC-3' R: 5'- AAGAAGTGCAGCGTCTGGTTTAC-3'	AY329356