Reproductive protein protects functionally sterile honey bee workers from oxidative stress

Siri-Christine Seehuus*, Kari Norberg*, Ulrike Gimsa†, Trygve Krekling‡, and Gro V. Amdam*§¶

Departments of *Animal and Aquacultural Sciences and ‡Plant and Environmental Sciences, Microscopy Division, Norwegian University of Life Sciences, N-1432 Ås, Norway; †Department of Neurology, University of Rostock, D-18147 Rostock, Germany; and §School of Life Sciences, Arizona State University, Tempe, AZ 85287

Edited by Mary Jane West-Eberhard, University of Costa Rica, Ciudad Universitaria, Costa Rica, and approved December 6, 2005 (received for review March 31, 2005)

Research on aging shows that regulatory pathways of fertility and senescence are closely interlinked. However, evolutionary theories on social species propose that lifelong care for offspring can shape the course of senescence beyond the restricted context of reproductive capability. These observations suggest that control circuits of aging are remodeled in social organisms with continuing care for offspring. Here, we studied a circuit of aging in the honey bee (*Apis mellifera*). The bee is characterized by the presence of a long-lived reproductive queen caste and a shorter-lived caste of female workers that are life-long alloparental care givers. We focus on the role of the conserved yolk precursor gene *vitellogenin* that, in *Caenorhabditis elegans*, shortens lifespan as a downstream element of the insulin/insulin-like growth factor signaling cascade. Vitellogenin protein is synthesized at high levels in honey bee queens and is abundant in long-lived workers. We establish that *vitellogenin* gene activity protects worker bees from oxidative stress. Our finding suggests that one mechanistic explanation for patterns of longevity in bees is that a reproductive regulatory pathway has been remodeled to extend life. This perspective is of considerable relevance to research on longevity regulation that builds largely on inference from solitary model species.

aging | social evolution | vitellogenin | carbonylation | RNA interference

Studies of pathways that regulate longevity have identified endocrine signaling cascades that control fecundity and aging (1–4). Insulin/insulin-like growth factor signaling (IIS) is one such conserved pathway (5). In the nematode *Caenorhabditis elegans*, IIS affects fertility and senescence antagonistically by means of separate regulatory events in the larval and adult stages, respectively (6). In *Drosophila melanogaster*, IIS increases adult reproductive capacity through a positive effect on ovary size during larval development and reduces lifespan in adult female flies (1, 7). Life-history regulation by IIS remains to be studied in eusocial organisms (8). However, eusocial insect females lack the negative association between fecundity and aging that is observed in solitary species (9, 10). The diverging female forms, or reproductive castes, that characterize eusocial insect societies appear to have evolved by means of reorganization (11) of regulatory circuits that controlled life-cycle stages in a solitary ancestor (12–15). This evolutionary view suggests that signaling cascades of development (11, 15), reproduction (12–14), and longevity (8) have been remodeled during social insect evolution.

The eusocial honey bee has the following two female forms: the long-lived reproductive queen that is physiologically specialized for oviposition and the facultatively sterile worker that displays flexible patterns of longevity linked to the remaining repertoire of female functions (reviewed in ref. 16). The queen may produce >1,500 eggs daily (17) and survive for 1–5 years (18). Workers may live up to 10 months (19–21), but lifespans are typically 4–6 weeks (17). Short lifespans emerge under favorable conditions because worker bees switch from nest activities to foraging duties after 2–3 weeks of adult life and normally survive only 1–3 weeks of active foraging (ref. 22 and references therein). However, ontogeny responds flexibly to changes in the intracolonial and extracolonial environment (23), enabling workers to nurse brood (eggs, larvae, and pupae) continuously until >130 days of age (24) or initiate foraging flights as early as 4–7 days after emergence from pupation (ref. 22 and references therein).

Aging in the bee may be influenced by juvenile hormone (JH) (9, 22, 25), and it has been proposed that the evolution of longevity regulation in workers was linked to exploitation of the conserved yolk precursor gene *vitellogenin* (25–27). JH is part of the IIS pathway in *Drosophila* (5) that coregulates yolk peptide expression and somatic maintenance antagonistically (1, 4, 28–30). In *C. elegans*, reduced activity of the vitellogenin-encoding genes *vit*-2 and *vit*-5 (which are downstream of the IIS receptor Daf-2) lengthens lifespan (3). Therefore, the aging machineries of the nematode, fly, and bee have similar components. However, there is a positive correlation between yolk precursor gene activity and longevity in the bee. The 180-kDa glycolipoprotein vitellogenin (31) is continuously synthesized at high levels in mature queens (including periods when they do not lay eggs) (32–34), and it likewise accumulates in long-lived workers (35). The regulatory action of JH on yolk precursor production is also uncommon (36) because high JH levels do not stimulate synthesis in adult females (34) but, rather, repress vitellogenin production during the workers' foraging stage (37).

A causal link between honey bee *vitellogenin* activity and lifespan is supported by the zinc-binding capacity of the protein product (25), which suggests antioxidant function (26, 38, 39). Oxidative modification of intracellular proteins is a major aspect of senescence (40, 41), and loss of oxidative stress resistance is a biomarker of aging in the nematode, fly, and mouse (5). Thus, the role of *vitellogenin* in honey bee aging could deviate substantially from the effect of yolk precursor gene activity (3) in the aging machineries (4, 5, 28, 30) of the distantly related solitary invertebrates that have been studied. However, a positive connection between *vitellogenin* activity and longevity first has to be established.

Here, we investigated whether a pathway of aging in a eusocial organism diverges from the current model in the published literature (3, 30) by testing the hypothesis that vitellogenin protects the bee from oxidative stress. Workers were studied because a *vitellogenin* RNA interference (RNAi) method is established for this caste (42). By using natural variation in the hemolymph vitellogenin level in addition to mRNA down-regulation by RNAi, we found that *vitellogenin* activity protects the bee against the oxidative damage agent paraquat, which induces formation of reactive oxygen species (43). The study also confirms that vitellogenin is preferentially oxidized, which is a

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: IIS, insulin/insulin-like growth factor signaling; JH, juvenile hormone; RNAi, RNA interference; hROS, highly reactive oxygen species.

¶To whom correspondence should be addressed. E-mail: gro.amdam@asu.edu.

www.pnas.org/cgi/doi/10.1073/pnas.0502681103

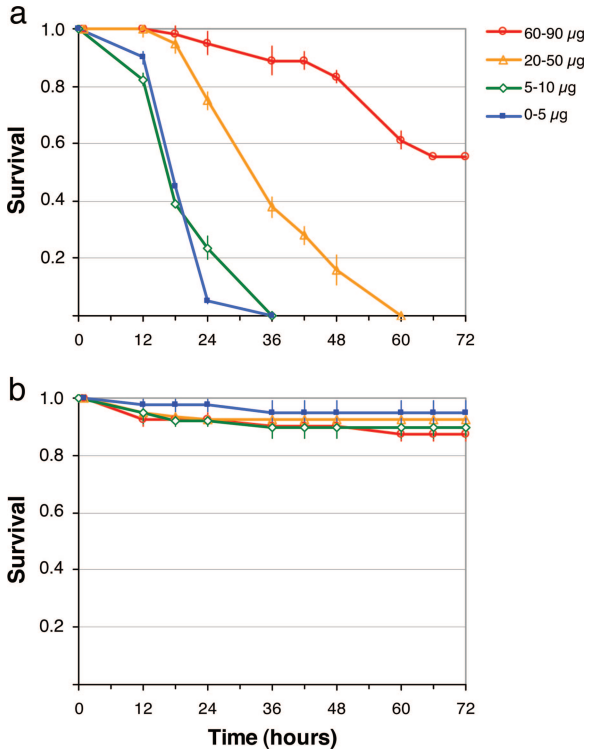

Fig. 1. Oxidative stress resistance is associated with the vitellogenin concentration in worker bees. (*a*) Survival after injections with paraquat for groups with naturally occurring differences in intrinsic vitellogenin levels in micrograms (per microliter of hemolymph). (*b*) Survival of groups after control injections with vehicle (nuclease-free water). Means ± SD for each group were obtained from independent replicates of the experiment ($n = 3$). The initial sample size of bees was constant between groups and trials ($n = 20$). Note that 56 ± 0.5% of the workers with the highest vitellogenin levels were resistant to the injected 150-μg/g (wt/wt) paraquat, which is ≈30-fold higher than the lethal dose in mammals.

property that is indicative of antioxidant function (39, 44, 45). We suggest that a changed connection between *vitellogenin* activity and aging in the bee can be understood by the role of decoupling of caste physiologies in social insect evolution (12, 13) and selection on longevity in species with continuing care for offspring (46–48). Specifically, life-history evolution in the bee, which provides continuing care via alloparents stemming from a single reproductive female, favored a positive link between *vitellogenin* activity and lifespan over the negative association (3, 30) we assume is the ancestral condition.

Results

We first determined whether the vitellogenin concentration of worker hemolymph (blood) was correlated with the level of oxidative stress resistance in a commercial stock of bees. Workers of commercial source show phenotypic variation for several traits including the vitellogenin titer (25), and, thus, the setup was designed as an initial assessment of putative association. We found that survival of workers with diverging hemolymph vitellogenin levels was significantly different after injections of paraquat ($\chi^2 = 137.8$, df $= 3$, $P < 0.0001$). Also, survival was positively associated with the intrinsic hemolymph titer of vitellogenin (Fig. 1*a*). Survival between groups that received control injections was not significantly different ($\chi^2 = 3.4$, df $= 3$, $P < 0.33$) (Fig. 1*b*).

Next, we turned off the effect of the *vitellogenin* gene to test specifically whether expression was linked to the bees' level of resistance to oxidative stress. To this end, we used RNAi to

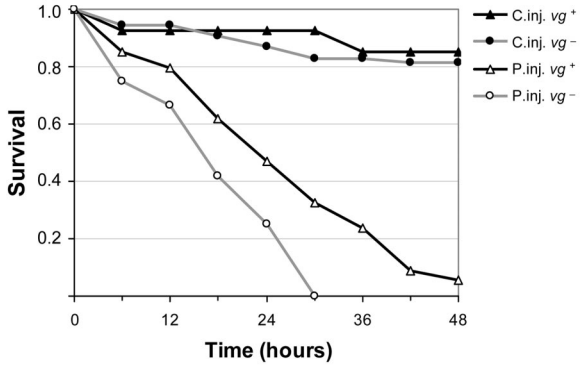

Fig. 2. Oxidative stress resistance is linked to *vitellogenin* gene activity in worker bees. Survival of vg^- and vg^+ phenotypes after treatment with vehicle (control injections, C. inj.) or paraquat (paraquat injections, P. inj.). Data were obtained from independent setups ($n = 2$), with initial total sample sizes of $n =$ 70 and 108, respectively. Stratifying the survival analysis to control for putative effects of replicate did not influence the treatment P values. Proportions of surviving workers are shown as the means of the two replicates.

decrease the amount of *vitellogenin* mRNA (42). Subsequent treatment of vg^- and vg^+ phenotypes with paraquat showed that survival was significantly lower for vg^- bees ($F_{72,64} = 2.2$, $P < 0.001$), whereas vg^- and vg^+ workers that were injected with vehicle survived with equal probability ($F_{20,16} = 1.3$, $P < 0.30$) (Fig. 2). The data show that *vitellogenin* activity is causally linked to the oxidative stress resistance of the bee.

To verify that honey bee vitellogenin has properties that are indicative of antioxidant function, we next investigated whether paraquat preferentially induced oxidative damage to this protein. Oxidative damage to vitellogenin, apolipoprotein 1, and hexamerin was analyzed by immunodetection of oxidative carbonylation (38). By using semiquantitative densitometry, we found that the protein carbonylation intensity per quantity was different ($F_{2,171} = 8.7$, $P < 0.0005$) and that vitellogenin was significantly more oxidized than apolipoprotein 1 and hexamerin (see Fig. 3 for statistics). Oxidation of vitellogenin, apolipoprotein 1, and hexamerin was significantly negatively correlated with the vitellogenin quantity (see Fig. 4*a* for information on P and

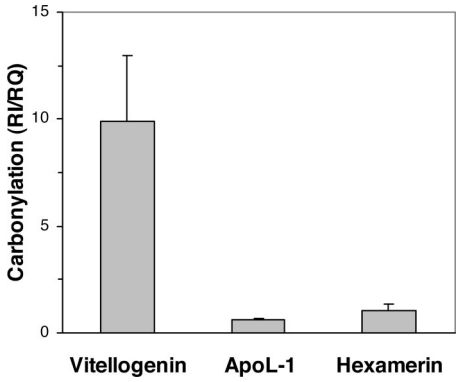

Fig. 3. Vitellogenin is targeted for oxidative modification. Measured as oxidative carbonylation intensity per quantity ($n = 58$), vitellogenin was oxidized more strongly than apolipoprotein 1 (ApoL-1) ($P < 0.0005$) and hexamerin ($P < 0.001$). The carbonylation intensities per quantity of apolipoprotein 1 and hexamerin were not different ($P = 0.85$). Carbonylation is expressed in relative intensities (RI) after normalization to the mean densitometrical intensity, and protein amounts are measured in relative quantities (RQ) after normalization to the mean integrated intensity of apolipoprotein 1. P values were obtained by using the least significant difference post hoc test. SEs are presented on top of each bar.

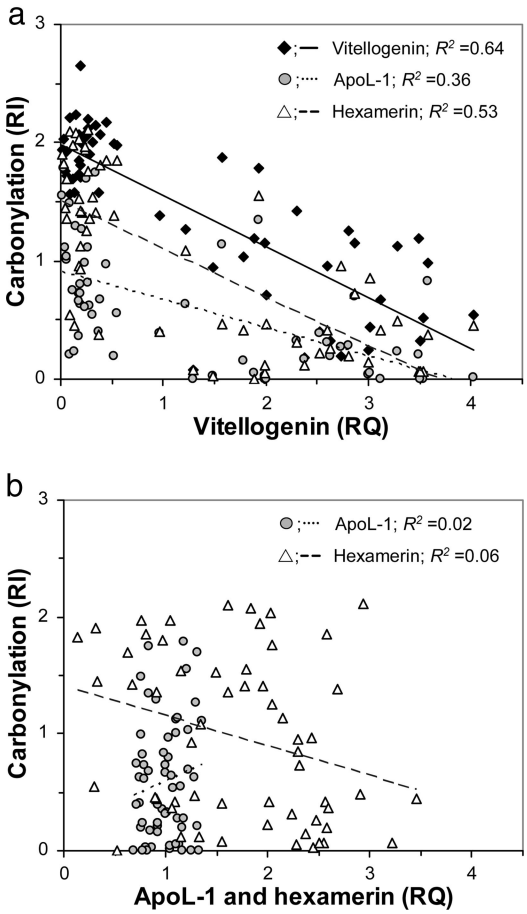

Fig. 4. Vitellogenin level explains variation in oxidative damage. (*a*) Individual carbonylation intensities of vitellogenin, apolipoprotein 1 (ApoL-1) and hexamerin plotted against the vitellogenin quantity of each sample ($n = 58$). Damage to proteins was negatively correlated with the vitellogenin level ($P < 0.00001$ for each correlation). (*b*) Individual carbonylation intensities of apolipoprotein 1 and hexamerin plotted against their corresponding protein quantities ($n = 58$). Here, protein quantities do not explain variation in carbonylation ($P = 0.29$ and 0.06 for apolipoprotein 1 and hexamerin, respectively). Carbonylation is expressed in relative intensities (RI) after normalization to the mean densitometrical intensity, and protein amounts are measured in relative quantities (RQ) after normalization to the mean integrated intensity of the apolipoprotein 1 band. Therefore, relative quantities of apolipoprotein 1 cluster around a value of 1 on the *x* axis of the lower panel. *P* and R^2 values were obtained from a Pearson correlation analysis.

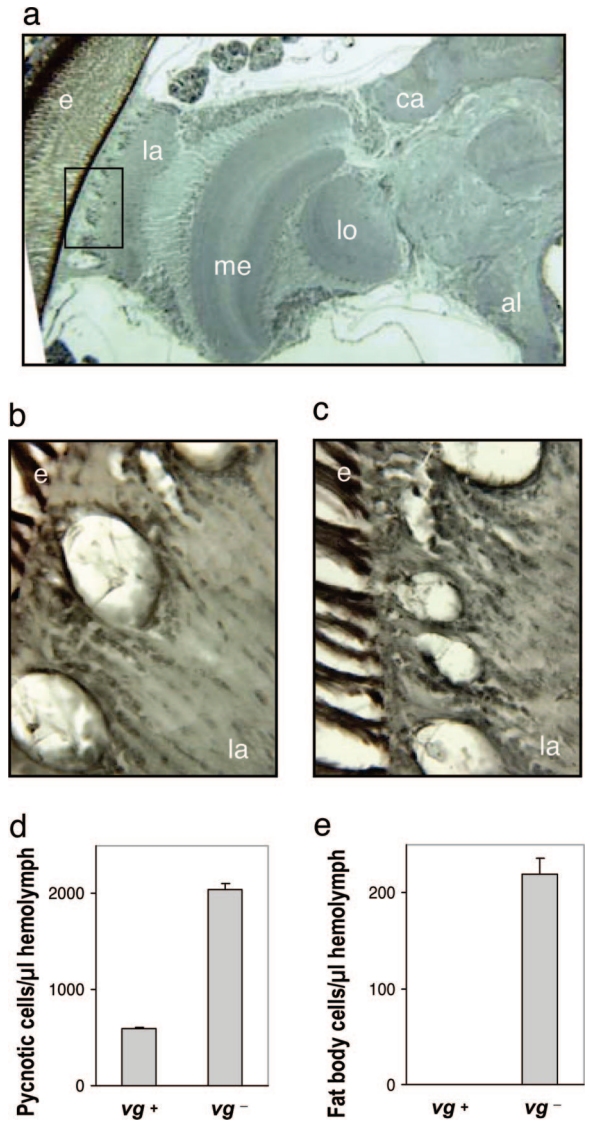

Fig. 5. Oxidative stress induced by paraquat has heterogeneous physiological effects. (*a*) Dinitrophenyl staining of honey bee brain. Frontal section of a bee brain at ×5 magnification. The box indicates the areas depicted at ×63 magnification for a paraquat-injected bee (*b*) and a control (*c*). The sections show no difference in positive (black) staining for oxidative protein carbonylation. e, eye; la, lamina; me, medulla; lo, lobula; ca, calyx; al, antennal lobe. (*d*) Elevated numbers of pycnotic immunocytes ($n = 32$). (*e*) Occurrence of dissociated fat body cells in the vg^- phenotype ($n = 32$). SEs are presented on top of each bar.

R^2 values), and more variation in oxidation of apolipoprotein 1 and hexamerin was explained by the hemolymph quantity of vitellogenin than by the hemolymph quantities of apolipoprotein 1 and hexamerin (compare statistics in Fig. 4 *a* and *b*). Thus, vitellogenin was preferentially oxidized, and lower concentrations were linked to stronger carbonylation of the other proteins that were examined. These findings substantiate that vitellogenin has an antioxidant function (44, 45).

Last, to examine the physiological link between *vitellogenin* activity, oxidative damage, and mortality, we tested whether paraquat exposure caused neurodegeneration as it does in humans (49) and *Drosophila* (50). Data were obtained by immunohistochemical staining for differential carbonylation and nitration damage, in addition to detection of putative apoptosis in brain tissue. These assays were negative (e.g., Fig. 5 *a–c*). We then screened the hemolymph for physiological markers and found that immunocytes from vg^- bees produced highly reactive oxygen species (hROS) at levels that were not different from cells reacted with 30% H_2O_2 ($P = 0.58$; see *Materials and Methods* for details on the ANOVA). This level was significantly different from that of vg^+ controls ($P < 0.005$). We also found elevated numbers of pycnotic immunocytes ($t_{31} = 6.4, P < 0.005$) (Fig. 5*d*) along with free-floating fat body cells in vg^- workers ($t_{31} = 3.1, P < 0.005$) (Fig. 5*e*). The fat body is analogous to the mammalian liver and white adipose tissue, and is implicated in extending lifespan by altered IIS (7). Break-up of the fat body actin cytoskeleton by exposure to paraquat (51) further implies that vital physiological functions (52) are lost. Thus, our results indicate that excess mortality of vg^- bees was linked to cellular damage that included a severe oxidative insult to the fat body.

Discussion

We have shown that *vitellogenin* activity protects worker bees from oxidative stress. This function was initially supported by

survival patterns in groups of bees of diverse ages, physiologies and behaviors. Previous work has documented that JH is one factor that varies between the groups that were used in this part of our study, because the JH titer is low at emergence and high at onset of foraging (reviewed in ref. 53). JH increases female reproduction, reduces tolerance to oxidative stress, and shortens lifespan in the fly (4, 30). Also, ablation of *Drosophila* insulin peptide synthesizing cells reduces fertility, elevates oxidative stress resistance, and extends longevity (54). *Drosophila* insulin peptides travel along the medial secretory neuron to the corpus cardiacum that projects to the JH-producing corpus allatum (ref. 55 and references therein). These findings suggest that gonotrophic JH signaling that is controlled by *Drosophila* insulin peptides regulates oxidative stress resistance and lifespan in the fly (5, 55). Here, our data from the bee are of interest (Fig. 1*a*), because the dynamics of the JH titer in honey bee workers (reviewed in ref. 53) imply that the hormone level was not only low in the group identified as highly tolerant to paraquat (nonforaging workers with vitellogenin levels of 60–90 μg/μl), but also in bees of intermediate and strong susceptibility (nursing and newly emerged workers with vitellogenin levels of 20–50 and 0–5 μg/μl, respectively). Thus, although our results suggest that honey bees with high JH titers are susceptible to oxidative stress (foragers with vitellogenin levels of 5–10 μg/μl), the data reveal tolerance patterns that are better explained by diverging vitellogenin levels than by JH signaling.

We confirmed that *vitellogenin* activity has a positive effect on oxidative stress resistance by studies of the survival and physiology of vg^- and vg^+ bees obtained with an RNAi protocol. Also, we showed that vitellogenin is a preferred target of oxidative carbonylation in comparison with apolipoprotein 1 and hexamerin, which similar to vitellogenin are major hemolymph proteins in adult bees (56–58). Preferential carbonylation has been described for Cu,Zn-superoxide dismutase (59, 60), a key antioxidant enzyme with metal-binding characteristics that are comparable with properties of vitellogenin. Also, versatile roles of vitellogenin in hemolymph have been compared with functions of serum albumins (25). Albumin is a metal-binding protein that can act as a free-radical scavenger and decrease levels of oxidative markers, such as protein carbonyls (reviewed in ref. 39). Most important, however, a positive effect of *vitellogenin* activity on longevity is supported by a consistent link between life extension and tolerance to oxidative stress in invertebrate- and vertebrate model species (ref. 54 and references therein).

The protein structure of vitellogenin is largely conserved across oviparous taxa (61). However, with respect to the effect of *vitellogenin* gene activity on lifespan, the honey bee stands out as a clear exception when compared with the distantly related invertebrates that have been studied. In general terms, yolk precursor-encoding genes are downstream of signaling cascades that shorten lifespan in solitary model organisms (4, 28, 54). IIS is necessary for vitellogenesis in *Drosophila* (29), and negative effects on longevity have been shown directly for vitellogenin-encoding genes that are controlled by IIS in *C. elegans* (3).

We propose that evolutionary segregation and subsequent specialization of reproductive and alloparental female forms (12) provided a context that selected for a remodeling of the relationship between *vitellogenin* gene activity and lifespan in the bee. The developmental trajectories of honey bee queens and workers are decoupled during the larval stage by an endocrine switch (reviewed in ref. 62). Evolution of this bifurcation enabled independent selection on the reproductive and alloparental phenotypes (12). Thus, traits that are associated with oviposition could be amplified in queens while being reciprocally deleted in workers, as described by West-Eberhard (12). Evolution of social insect castes with diverging reproductive capability also provided a context for selection on the traits that affected longevity (63). From this perspective, the life history of the honey bee suggests that a positive association between vitellogenesis and lifespan emerged through independent selection on the queen caste. This condition occurred because both the fecundity and longevity of the queen (17) underlie the social continuum that allows colonies to repeatedly build up to a size where reproduction by fission (swarming) and drone (male) production is possible (comparative information in ref. 64). Thus, the bee would exemplify (63) that, by social evolution (46, 47, 65), reproductive control circuits can be remodeled to extend life. However, this proposition rests on the assumption that the solitary ancestor of the honey bee was characterized by an antagonistic relationship between *vitellogenin* activity and longevity. Therefore, studies of closely related taxa of diverse life histories must be conducted to elucidate the ancestral mode of aging regulation and the molecular evolution of honey bee longevity determination.

The hypothesis that a positive association between *vitellogenin* activity and lifespan evolved in the bee by selection on the queen caste is supported by vitellogenin dynamics and patterns of longevity in workers. Specifically, a worker phenotype was identified that, in response to reduced brood rearing, can accumulate vitellogenin to queen-like levels of 50–60% of the hemolymph protein fraction (32, 35) and survive for 6–10 months (16, 19–21). The ability to develop this facultative worker form is characteristic of temperate subspecies *Apis mellifera mellifera* and *Apis mellifera carnica* but is absent from subtropical *Apis mellifera scutellata* and neotropical *Apis mellifera scutellata* hybrids (66, 67). The honey bee evolved in tropical Africa and later migrated to temperate zones (68). It is likely that a long-lived worker phenotype emerged under this migration (16, 26, 66, 69), and thus, the form appeared subsequent to the above outlined life-history evolution that extended queen longevity. Also, utilization of *vitellogenin*-driven stress resistance in workers appears to build on preexisting patterns of vitellogenin production and usage. Worker bees synthesize vitellogenin during the nurse stage that precedes foraging (32). The protein is a source for brood food produced by the hypopharyngeal glands (paired acinous head glands), which express a vitellogenin receptor similar to that of the queen ovary (27). However, the rate of vitellogenin production in workers is low compared with queens (34). Thus, the protein may be depleted from nurse bees by brood feeding before it is suppressed by the JH increase that accompanies onset of foraging (26). This pattern is shared between the subspecies that have been studied (32, 34, 37, 66), which substantiates that absence of brood is a necessary, but not sufficient, condition for vitellogenin to build up to produce long-lived workers (66). Therefore, modular regulatory mechanisms of vitellogenin production (34) and homeostasis (66) may have been recombined (11, 70) between the queen and worker caste during the migration of the bee to temperate regions. With this proposition, we imply that genes and gene-regulatory elements that were selected to become coexpressed in the queen caste later shifted to become coexpressed conditionally also in workers. This recombination resulted in a long-lived, stress-resistant, and facultatively expressed alloparental phenotype that allowed colonies to survive the winter period when short-lived workers could not be replaced because of environmental constraints on brood rearing. In this context, the bee may show how circuits of aging can evolve when decoupled from reproduction, and thus, expand our understanding of the flexibility, modularity, and evolvability of longevity regulation (concepts in ref. 11).

Materials and Methods

Bees. In the commercial stocks of the *A. m. mellifera* and *A. m. carnica* hybrids used in this study, newly emerged bees have vitellogenin levels of 0–5 μg/μl hemolymph, 14- to 30-day-old foragers without wing wear have 5–10 μg/μl, 10- to 20-day-old nurse bees that care for larvae have 20–50 μg/μl, and 14- to

30-day-old nonforaging bees in colonies without brood have 60–90 μg/μl (25, 56). Thus, for our initial setup, newly emerged workers were obtained as described in ref. 56, sampled directly, or marked with Uni Posca paint. Marked bees were introduced into hives where the amount of brood was controlled (56). Marked nurse bees with their head and thorax in cells with larvae, and marked bees kept in absence of brood were collected at the relevant ages described above. Marked foragers were retrieved at the hive entrances.

In subsequent experiments, effects of *vitellogenin* activity were dissociated from confounding factors by establishing RNAi knockdowns (vg^-) and controls (vg^+), as described in ref. 42. Newly emerged workers were injected with 1 μl of dsRNA 5 μg/μl vehicle, or vehicle only (nuclease-free water, a control for *vitellogenin* RNAi) (42). The dsRNA was synthesized by RiboMax T7 (Promega) with DNA template from *vitellogenin* (GenBank accession no. AJ517411) clone *AP4a5*. Treatment groups were marked with separate color codes and introduced into laboratory colonies (71) with free access to pollen and sugar dissolved in distilled water 30% (wt/vol). Marked bees were collected at 7 days of age.

After newly emerged bees and marked retrieved workers were injected with paraquat (1,1′-dimethyl-4,4′-bipyridinium; Sigma-Aldrich) or vehicle, they were kept at 35°C in cages with sugar dissolved in distilled water 30% (wt/vol) and pollen dough. Cages were monitored for nonviable bees (lying on their back with extended proboscis) every 6 h. A sample of vg^- and vg^+ bees was removed for quantification of vitellogenin levels by using 7% SDS/PAGE and β-galactosidase standard (Sigma-Aldrich) as reported in ref. 56. The assay confirmed the following knockdown and control phenotypes: 1.2 ± 0.4 ($n = 19$) and 19.7 ± 3.3 μg/μl hemolymph ($n = 20$) for vg^- and vg^+ bees, respectively. The latter vitellogenin concentration is normal for 7-day-old bees (14, 56). Samples were collected from cages that were not part of the survival survey for semiquantitative analyses of protein quantities and carbonylation intensities, as well as examination of cell characteristics (see below).

Dosage and Injection. Paraquat is regularly given at doses of ≈5 μg/g in studies of oxidative stress in mammals (e.g., ref. 51). However, bees experience social stress when removed from a colony (72), and mortality due to isolation increased after 4–7 days in our cage setup. Thus, we used 150 μg/g per bee (wt/wt), which caused a large percentage of workers to die within 3 days. This protocol prevented confounding by social stress. Paraquat was dissolved in nuclease-free water. Controls were injected with vehicle. Injections were made between the fifth and sixth abdominal segments by using a microsyringe (Hamilton) as reported (42). The injection volume was 2 μl.

Semiquantitative Measurements of Oxidative Intensity and Protein Quantity. Oxidative stress damage to hemolymph proteins was assayed with OxyBlot kit (Intergen, Purchase, NY) for detection of modified carbonyl groups (i.e., oxidative carbonylation) (41). Protein carbonyls were derivatized to 2,4-dinitrophenylhydrazone and separated by 7% SDS/PAGE. Each lane was loaded with a sample of 1 μl of hemolymph from a single bee. Separated proteins were electroblotted onto immunoblot PVDF membranes (Bio-Rad) before incubation with a mAb to dinitrophenyl (Intergen), as described for OxyBlot. Blots were developed with diaminobenzidine-nickel solution (Sigma-Aldrich), which is a widely used chromogen, before oxidative damage was estimated densitometrically for vitellogenin (8), apolipoprotein 1 (58), and hexamerin (Hex 70a; ref. 57) with QUANTITY ONE imaging software (Bio-Rad). Blot-to-blot variation in staining intensity was controlled by background correction and internal standard (corresponding samples transferred to each blot). To obtain corresponding semiquantitative measurements of protein quantities, the procedure was also used to estimate integrated intensities of bands on duplicate gels stained with Coomassie brilliant blue dye reagent (Sigma-Aldrich).

Analysis of Oxidative Damage in Tissues. Bees were fixed in 4% methanol-free formaldehyde (Polysciences) with 0.2% Triton X-100 (Sigma-Aldrich). Samples were cut in 20-μm cryosections or infiltrated with LR-White (Electron Microscopy Sciences, Fort Washington, PA) and cut in semithin sections. Sections were stained by using mAbs to dinitrophenyl (1:150) (Intergen) for detection of oxidative carbonylation or to nitrotyrosine (1:200) (Chemicon) for detection of oxidative nitration damage. Sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:300) and horseradish peroxidase (1:200) (Sigma-Aldrich), developed with diaminobenzidine/nickel solution, mounted in DePex (Electron Microscopy Sciences), and examined by light microscopy.

TUNEL Staining. Fluorometric TUNEL staining was conducted as described for the DeadEnd Fluorometric TUNEL system (Promega), which identifies apoptotic cells by fluorescein-12–dUTP labeling of fragmented DNA.

hROS and Cells in Hemolymph. Hydroxyphenyl fluorescein hROS indicator (Molecular Probes) was diluted to 10 μM in Grace insect medium (Invitrogen). Hemolymph (5 μl) was extracted as described (42) and transferred to wells with 100 μl of staining solution. As control, 5 μl of hemolymph was reacted with 1 μl of 30% H_2O_2 before staining. Samples were incubated at 35°C for 1 h and analyzed in a Victor 3TM 1420 multilabel counter (PerkinElmer). Cells in hemolymph were counted and characterized in 0.05-mm Bürker cell counters (Karl Hecht, Sondheim, Germany) after staining in phosphate-buffered formaldehyde (4%) with 1 g/liter methylene blue (Sigma-Aldrich), as described in ref. 56.

Data Analysis. We used the multiple-sample extension of Gehan's generalized Wilcoxon test to examine whether survival of bees with diverging vitellogenin levels was different after injections of paraquat or vehicle. Survival of vg^- and vg^+ phenotypes was studied by using Cox's F test for two groups. Stratified Cox's regression analysis was used to test for influence of replicates. To analyze carbonylation intensity per integrated intensity (i.e., per quantity) of vitellogenin, apolipoprotein 1, and hexamerin, we used one-way ANOVA, followed by least significant difference post hoc comparison. Pearson correlation analysis was used to examine relationships between carbonylation intensities and protein quantities. Carbonylation intensities were expressed on a relative scale after normalization to the overall mean carbonylation intensity. Integrated intensities of protein bands were expressed as relative quantities after normalization to the mean integrated intensity of apolipoprotein 1, which is present at close to constant levels in adult bees (58). Production of hROS by immunocytes was analyzed by one-way ANOVA, in which treatment levels were assigned the following categorical factors: 1, cells from noninjected bees; 2, cells from bees injected with vehicle; 3, cells from bees injected with paraquat; and 4, cells reacted with 30% H_2O_2. The initial test was positive (ANOVA; $F_{3,52} = 3.9$, $P = 0.01$) and was followed by least significant difference post hoc comparisons of treatment levels. Differences in numbers of pycnotic immunocytes (immune cells undergoing cell death) (25, 73) and dissociated fat body cells in hemolymph from vg^- and vg^+ bees were tested with Student's t test. All statistical analyses were performed with STATISTICA (Version 6.0).

We thank C. Brent, J. Carey, J. Harrison, K. Hartfelder, G. Hunt, G. Klein, C. Murphy, R. Scheiner, R. E. Page, and two referees for helpful reviews of the manuscript; the editor for insights into the evolution of longevity; and Z. L. P. Simões (Ribeirão Preto, Brazil) for clone *AP4a5*.

This work was supported by Norwegian Research Council Grants 147085/110 and 157851/432 (to S.-C.S. and G.V.A.), National Institute on Aging Grant P01 AG22500 (to G.V.A.), and German Ministry of Education and Research Grant 01ZZ0108 (to U.G.).

1. Tu, M. P. & Tatar, M. (2003) *Aging Cell* **2,** 327–333.
2. Rea, S. & Johnson, T. E. (2003) *Dev. Cell* **5,** 197–203.
3. Murphy, C. T., McCarroll, S. A., Bargmann, C. I., Fraser, A., Kamath, R. S., Ahringer, J., Li, H. & Kenyon, C. (2003) *Nature* **424,** 277–284.
4. Tatar, M., Chien, S. A. & Priest, N. K. (2001) *Am. Nat.* **158,** 248–258.
5. Tatar, M., Bartke, A. & Antebi, A. (2003) *Science* **299,** 1346–1350.
6. Dillin, A., Crawford, D. K. & Kenyon, C. (2002) *Science* **298,** 830–834.
7. Giannakou, M. E., Gross, M., Jünger, M. A., Hafen, E., Leevers, S. J. & Partridge, L. (2004) *Science* **305,** 361.
8. Amdam, G. V. (2005) in *Longevity and Frailty*, eds. Carey, J. R., Robine, J.-M., Michel, J.-P. & Christen, Y. (Springer, Berlin), pp. 17–26.
9. Finch, C. E. (1990) *Longevity, Senescence, and the Genome* (Univ. of Chicago Press, Chicago).
10. Hartmann, A. & Heinze, J. (2003) *Evolution (Lawrence, Kans.)* **57,** 2424–2429.
11. West-Eberhard, M. J. (2003) *Developmental Plasticity and Evolution* (Oxford Univ. Press, New York).
12. West-Eberhard, M. J. (1996) in *Natural History and Evolution of Paper Wasps*, eds. Turillazzi, S. & West-Eberhard, M. J. (Oxford Univ. Press, New York), pp. 290–317.
13. West-Eberhard, M. J. (1987) in *Animal Societies: Theories and Fact*, eds. Itô, Y., Brown, J. L. & Kikkawa, J. (Japan Sci. Soc. Press, Tokyo), pp. 35–51.
14. Amdam, G. V., Norberg, K., Fondrk, M. K. & Page, R. E. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 11350–11355.
15. Hunt, J. H. & Amdam, G. V. (2005) *Science* **308,** 264–267.
16. Omholt, S. W. & Amdam, G. V. (2004) *Sci. Aging Knowledge Environ.* **26,** pe28.
17. Winston, M. L. (1987) *The Biology of the Honey Bee* (Harvard Univ. Press, Cambridge, MA).
18. Page, R. E. & Peng, C. Y.-S. (2001) *Exp. Gerontol.* **36,** 695–711.
19. Farrar, C. L. (1949) *Bee World* **30,** 51.
20. Maurizio, A. (1950) *Bee World* **31,** 9–12.
21. Fukuda, H. & Sekiguchi, K. (1966) *Jap. J. Ecol.* **16,** 206–212.
22. Amdam, G. V. & Omholt, S. W. (2003) *J. Theor. Biol.* **223,** 451–464.
23. Huang, Z.-Y. & Robinson, G. E. (1996) *Behav. Ecol. Sociobiol.* **39,** 147–158.
24. Haydak, M. H. (1963) *Minn. Agric. Exp. Sta. Sci. J. Ser.* **5122,** 101–103.
25. Amdam, G. V., Simões, Z. L. P., Hagen, A., Norberg, K., Schrøder, K., Mikkelsen, O., Kirkwood, T. B. L. & Omholt, S. W. (2004) *Exp. Gerontol.* **39,** 767–773.
26. Amdam, G. V. & Omholt, S. W. (2002) *J. Theor. Biol.* **216,** 209–228.
27. Amdam, G. V., Norberg, K., Hagen, A. & Omholt, S. W. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 1799–1802.
28. Tatar, M. & Yin, C. M. (2001) *Exp. Gerontol.* **36,** 723–738.
29. Richard, D. S., Rybczynski, R., Wilson, T. G., Wang, Y., Wayne, M. L., Zhou, Y., Partridge, L. & Harshman, L. G. (2005) *J. Insect Physiol.* **51,** 455–464.
30. Salmon, A. B., Marx, D. B. & Harshman, L. G. (2001) *Evolution (Lawrence, Kans.)* **55,** 1600–1608.
31. Wheeler, D. E. & Kawooya, J. K. (1990) *Arch. Insect Biochem. Physiol.* **14,** 253–267.
32. Engels, W. & Fahrenhorst, H. (1974) *Roux's Arch.* **174,** 285–296.
33. Fluri, P., Sabatini, A. G., Vecchi, M. A. & Wille, H. (1981) *J. Apic. Res.* **20,** 221–225.
34. Engels, W., Kaatz, H., Zillikens, A., Simões, Z. L. P., Truve, A., Braun, R. P. & Dittrich, F. (1990) in *Advances in Invertebrate Reproduction*, eds. Hoshi, M. & Yamashita, O. (Elsevier Science, Amsterdam), Vol. 5, pp. 495–502.
35. Fluri, P., Lüscher, M., Wille, H. & Gerig, L. (1982) *J. Insect Physiol.* **28,** 61–68.
36. Hartfelder, K. (2000) *Braz. J. Med. Biol. Res.* **33,** 157–177.
37. Pinto, L. Z., Bitondi, M. M. G. & Simões, Z. L. P. (2000) *J. Insect Physiol.* **46,** 153–160.
38. Goto, S., Nakamura, A., Radak, Z., Nakamoto, H., Takahashi, R., Yasuda, K., Sakurai, Y. & Ishii, N. (1999) *Mech. Ageing Dev.* **107,** 245–253.
39. Quinlan, G. J., Martin, G. S. & Evans, T. W. (2005) *Hepatology* **41,** 1211–1219.
40. Finkel, T. & Holbrook, N. J. (2000) *Nature* **408,** 239–247.
41. Nystrom, T. (2005) *EMBO J.* **24,** 1311–1317.
42. Amdam, G. V., Simões, Z. L. P., Guidugli, K. R., Norberg, K. & Omholt, S. W. (2003) *BMC Biotechnol.* **3,** 1–8.
43. Day, B. J., Patel, M., Calavetta, L., Chang, L. Y. & Stamler, J. S. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 12760–12765.
44. Salo, D. C., Lin, S. W., Pacifici, R. E. & Davies, K. J. A. (1988) *Free Radical Biol. Med.* **5,** 335–339.
45. Andrus, P. K., Fleck, T. J., Gurney, M. E. & Hall, E. D. (1998) *J. Neurochem.* **71,** 2041–2048.
46. Lee, R. D. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 9637–9642.
47. Lahdenperä, M., Lummaa, V., Helle, S., Tremblay, M. & Russell, A. F. (2004) *Nature* **428,** 178–181.
48. Rogers, A. R. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 9114–9115.
49. Teixeira, H. D., Schumacher, R. I. & Meneghini, R. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 7872–7875.
50. Pesah, Y., Pham, T., Burgess, H., Middlebrooks, B., Verstreken, P., Zhou, Y., Harding, M., Bellen, H. & Mardon, G. (2004) *Development (Cambridge, U.K.)* **131,** 2183–2194.
51. Cappelletti, G., Inciani, C. & Maci, R. (1994) *Cell Biol. Toxicol.* **10,** 255–263.
52. Jensen, P. V. & Borgesen, L. W. (2000) *Arthr. Struct. Dev.* **29,** 171–184.
53. Bloch, G., Wheeler, D. & Robinson, G. E. (2002) in *Hormones, Brain and Behavior*, eds. Pfaff, D., Arnold, A., Etgen, A., Fahrbach, S., Moss, R. & Rubin, R. (Academic, San Diego), Vol. 3, pp. 195–235.
54. Broughton, S. J., Piper, M. D. W., Ikeya, T., Bass, T. M., Jacobson, J., Driege, Y., Martinez, P., Hafen, E., Withers, D. J., Leevers, S. J. & Partridge, L. (2005) *Proc. Natl. Acad. Sci. USA* **102,** 3105–3110.
55. Tatar, M. (2004) *Exp. Gerontol.* **39,** 1745–1750.
56. Amdam, G. V., Hartfelder, K., Norberg, K., Hagen, A. & Omholt, S. W. (2004) *J. Econ. Entomol.* **97,** 741–747.
57. Danty, E., Arnold, G., Burmester, T., Huet, J. C., Huet, D., Pernollet, J. C. & Masson, C. (1998) *Insect Biochem. Mol. Biol.* **28,** 387–397.
58. Shipman, B. A., Ryan, R. O., Schmidt, J. O. & Law, J. H. (1987) *Biochemistry* **26,** 1885–1889.
59. Kwon, O. J., Lee, S. M., Floyd, R. A. & Park, J. W. (1998) *Biochim. Biophys. Acta* **1387,** 249–256.
60. Choi, J., Rees, H. D., Weintraub, S. T., Levey, A. I., Chin, L. S. & Li, L. (2005) *J. Biol. Chem.* **280,** 11648–11655.
61. Spieth, J., Nettleton, M., Zuckeraprison, E., Lea, K. & Blumenthal, T. (1991) *J. Mol. Evol.* **32,** 429–438.
62. Hartfelder, K. & Engels, W. (1998) *Curr. Top. Dev. Biol.* **40,** 45–77.
63. Keller, L. & Genoud, M. (1997) *Nature* **389,** 958–960.
64. Jeanne, R. L. (1991) in *The Social Biology of Wasps*, eds. Ross, K. G. & Matthews, R. W. (Cornell Univ. Press, Ithaca).
65. Amdam, G. V. & Page, R. E. (2005) *Aging Res. Rev.* **4,** 398–408.
66. Amdam, G. V., Norberg, K., Omholt, S. W., Kryger, P., Lourenco, A. P., Bitondi, M. M. G. & Simões, Z. L. P. (2005) *Insectes Soc.* **52,** 316–319.
67. Winston, M. L. (1980) *J. Kans. Entomol. Soc.* **53,** 157–165.
68. Ruttner, F. (1988) *Biogeography and Taxonomy of Honeybees* (Springer, Berlin).
69. Villa, J. D., Koeniger, N. & Rinderer, T. E. (1991) *Environ. Entomol.* **20,** 39–43.
70. West-Eberhard, M. J. (2005) *Proc. Natl. Acad. Sci. USA* **102,** 6543–6549.
71. Omholt, S. W., Hagen, A., Elmholdt, O. & Rishovd, S. (1995) *Apidologie* **26,** 297–304.
72. Huang, Z.-Y. & Robinson, G. E. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11726–11729.
73. Wille, H. & Rutz, W. (1975) *Schweiz. Landwirtschaft. Forsch.* **14,** 339–353.