Vitellogenin regulates hormonal dynamics in the worker caste of a eusocial insect

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Abstract Functionally sterile honey bee workers synthesize the yolk protein vitellogenin while performing nest tasks. The subsequent shift to foraging is linked to a reduced vitellogenin and an increased juvenile hormone (JH) titer. JH is a principal controller of vitellogenin expression and behavioral development. Yet, we show here that silencing of vitellogenin expression causes a significant increase in JH titer and its putative receptor. Mathematically, the increase corresponds to a dynamic dose–response. This role of vitellogenin in the tuning of the endocrine system is uncommon and may elucidate how an ancestral pathway of fertility regulation has been remodeled into a novel circuit controlling social behavior.

Keywords: Juvenile hormone; Vitellogenin; Ultraspiracle; Age polyethism; Honey bee

1. Introduction

Understanding the proximate mechanisms that underlie division of labor in eusocial insect societies is a major issue in sociobiology. In particular, the honey bee has emerged as a key model for dissecting the regulatory architecture that gives rise to task partitioning in social groups. Honey bee workers undergo a characteristic progression in task performance. They care for brood during the first two weeks of adult life (“nursing”), and later start foraging for nectar and pollen. This behavioral switch is accompanied by an increase in the juvenile hormone (JH) titer and a decrease in the vitellogenin protein level [1,2]. JH influences reproductive behavior and physiology in a broad range of insects [3] and vitellogenins are yolk precursor proteins in most oviparous taxa. The unconventional production pattern of JH and vitellogenin in worker bees [4] has, however, spurred the idea that this basic reproductive molecule may be part of the pathway that controls the behavioral shift to foraging in social insects with a functionally sterile worker caste [1].

Season and colony age demography influence the JH titer and, consequently, the timing of onset of foraging behavior in individual honey bee workers [5,6]. This plastic and adaptive individual level response to colony needs led to the perception that JH may function as a pacemaker of behavioral development in workers [7]. However, recent experiments on allatectomized bees [8] show that JH is not the only effector that drives the behavioral transition to foraging. Thus, a better understanding of the regulatory signature in the shift to foraging activity is required, also because insights into this signature may provide novel information on the evolutionary trajectory of pathways that control division of labor in social insects.

Regulation of female fertility in the social Hymenoptera is usually considered under the general paradigm of hormonal control of insect reproduction, where JH and ecdysteroids act as the main inducers of vitellogenin synthesis and uptake [3]. In adult honey bees, however, vitellogenin synthesis is not upregulated by these hormones [4], and it is only during the initiation of vitellogenin expression in the late pupal stages that JH acts as an inducer of vitellogenin production [9]. This unconventional association was partly explained when it was shown that vitellogenin has evolved functions beyond the restricted context of reproduction: putative vitellogenin receptors can be found in the royal jelly producing hypopharyngeal glands of workers, suggesting that vitellogenin is used to synthesize proteins that nurse bees feed to the larvae [10]. The conversion of a yolk protein to larval food proteins is compatible with the physiological condition of nurse bees, which have high vitellogenin and low JH titers [11].

Based on available data, general life history considerations, and theory on the generic principles of stable states, a dynamic model of the forager transition was recently presented [2]. The mathematical model proposed that the shift from nest tasks to foraging is controlled by a two-repressor constellation that involves a positive regulatory feedback loop between JH and vitellogenin. Modulation of the parameter values of this feedback loop permitted simulations of co-variance patterns between the JH and vitellogenin titer that are observed under natural and experimental conditions. The simulation results supported the experimentally verified inhibitory effect of an elevated JH titer on the hemolymph vitellogenin level [4,11], and thereby the hormone’s association with the shift in worker behavior from the performance of nest-tasks to foraging for

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pollen and nectar. This transition is a major behavioral change that is also accompanied by a reorganization of central nervous system structures involved in learning and memory formation [1,7].

Yet, in addition to supporting the inhibitory effect of JH on vitellogenin, the mathematical simulations further arrived at the non-intuitive prediction that forced repression of vitellogenin synthesis should trigger an increase in the JH titer, which subsequently causes the worker bee to become behaviorally and physiologically locked in the forager state. It is this combination of inhibitory interactions that constitutes the Double Repressor framework [2] for the regulation of a honey bee worker's physiology during its adult life cycle. Thus, the "Double Repressor" hypothesis has implications well beyond those for the specific physiology of social insects due to its prediction of a regulatory association between JH and vitellogenin that is not commonly part of the reproductive architecture of female insects.

In the present study, we experimentally tested whether vitellogenin indeed exhibits this regulatory function in honey bee workers. We used RNA interference (RNAi) technology to silence vitellogenin expression and investigated the effect on the JH titer. In addition, we monitored the expression level of a putative JH receptor [12]. The study was performed on two genetically different bee sources kept under distinct social conditions to assess the robustness of a putative regulatory relationship involving vitellogenin and JH.

2. Materials and methods

2.1. Bees

Frames of sealed worker brood were retrieved from *Apis mellifera* colonies maintained at the Apiaries of the Norwegian University of Life Sciences, Aas, Norway (*Apis mellifera carnica*) and of the University of São Paulo at Ribeirão Preto, Brazil (Africanized hybrids). Bees emerging from brood cells were marked. The Norwegian source bees were introduced into one-story hives that contained an egg-laying queen. The Brazilian source bees were kept queenless in groups of 25–35 bees in an incubator. They received a pollen/sugar diet that supports vitellogenesis [13]. In both setups, the bees were retrieved after seven days for hemolymph and fat body sampling. Control foragers represent a random sample of bees that were collected from flowers near the apiary.

Seven days after the start of the experiment, the bees were retrieved and anesthetized on ice for hemolymph sampling [14] and dissection. For JH quantification, 1–3 μl of each hemolymph sample were drawn into 500 μl acetonitrile and stored at −20 °C. The remaining hemolymph was stored at −20 °C for protein electrophoresis. RNA was extracted from abdominal carcasses by a TRIzol protocol.

2.2. Silencing of vitellogenin expression

Double-stranded honey bee vitellogenin RNA was prepared as previously described [15] following the protocol of the Promega RiboMax™ T7 system (Promega) and using as template the clone A*p4a5* that contains a partial sequence of the honey bee vitellogenin cDNA [16]. After phenol–chloroform extraction and heat treatment, the dsRNA was diluted with nuclease-free water to a final concentration of 5 μg/μl. Newly emerged workers were injected intra-abdominally with 1 μl dsRNA solution or 1 μl water (sham). Bees showing signs of hemolymph leakage after withdrawal of the needle were discarded.

2.3. SDS–PAGE and RT-PCR

Protein analysis was performed using samples of 1 μl hemolymph that were subjected to SDS–PAGE (7.5% gel) and stained with Coomassie Brilliant Blue. Gels were scanned and imported into tnimage (3.3.12a, Linux) to quantify staining intensity and size of the vitellogenin and apolipoprotein-I bands. The latter is expressed at constant levels throughout the adult life cycle and was used for normalization of vitellogenin levels.

RNA extracted from abdominal carcasses was used for first-strand reverse transcription. These cDNA samples were subjected to PCR amplification with primers specific for honey bee *vitellogenin* [17] and *Apis mellifera ultraspiracle* (*usp*) [12]. For normalization, we performed RT-PCR amplifications on a constitutively expressed *β-actin* gene of *A. mellifera*.

2.4. Juvenile hormone titer analysis

The JH extraction procedure followed a protocol established for honey bee hemolymph [18]. Briefly, 1 ml NaCl (0.9%) and 1 ml hexane were added to the acetonitrile extract. After vigorous vortexing, the phases were separated by centrifugation ($700 \times g$). The hexane phase was removed and the extraction was repeated twice. The pooled hexane phases were dried and the residue was redissolved in 50 μl toluene. Before starting the RIA, the solvent was removed by vacuum centrifugation.

The antiserum used in this study was diluted to 1:1250 in phosphate buffer supplemented with bovine serum albumin (0.1%) and rabbit immunoglobulin G (0.1%). The assays were performed with [10-^{3}H(N)]-juvenile hormone III (spec. activity 19.4 Ci/mmol, NEN Life Science Products, Boston), diluted in the phosphate buffer to 6000–6500 cpm/50 μl. Juvenile hormone III (Fluka) was used as non-radioactive ligand. Standard curves were set up to cover a 50 pg to 10 ng range. The RIA procedure followed the protocol established by Goodman et al. [19]. JH titers of unknown samples are expressed as JH-III equivalents (pg/μl hemolymph).

3. Results and discussion

We found that *vitellogenin* gene knockdown by RNAi was associated with a significant increase in the JH titer of worker bees. This effect was observed consistently in both genetic backgrounds and social conditions, although the response was more apparent in the Africanized *A. mellifera* hybrids (Fig. 1A and B). In addition to the quantification of vitellogenin protein by densitometric analysis of the corresponding SDS–PAGE band in the full set of hemolymph samples (47 bees of Norwegian origin and 47 Africanized honey bees), we also monitored the vitellogenin transcript levels by RT-PCR in a subset of fatbody mRNA samples. These results confirmed the vitellogenin knockdown phenotype (Fig. 1C and D). The observed association between the downregulation of vitellogenin and upregulation of JH provides strong evidence for the previously hypothesized [2] regulatory feedback constellation between vitellogenin and JH. Our data also suggest that the relationship is quite robust to variation in the genetic origin and the social milieu of worker bees.

We further explored the dynamic linkage between vitellogenin and JH at the level of individual bees (Fig. 2A). This analysis performed on data from Africanized *A. mellifera* hybrids showed a significant negative correlation between the vitellogenin titer and the JH level in bees with the knockdown phenotype ($r = -0.66$, $P = 0.02$). The control groups, which consisted of untreated and sham-injected bees, displayed no such association ($r = 0.04$, $P = 0.82$), but a significant non-linear component was apparent when JH was modeled as a function of the hemolymph concentration of vitellogenin ($r = 0.51$, $P = 0.01$). This interpretation is supported by the non-linear threshold shift observed in the JH level of vitellogenin knockdown bees (Fig. 2A and B), which all exhibited JH titers above the 200 pg/μl upper bound of the control group.