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SHORT COMMUNICATION

# Plasticity and robustness of protein patterns during reversible development in the honey bee (*Apis mellifera*)

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**Abstract** With age, worker honey bees normally proceed from performing activities inside the nest to foraging in the field, creating an age-related division of labor. We previously established that the whole-body protein profiles of nest workers and foragers are different, and proposed that this proteomic divergence in part is explained by a shift in metabolic requirements as worker bees initiate intense flight. The unique plasticity of honey bee worker ontogeny, however, provides further opportunities to investigate if such changes in the proteome are dynamic or, alternatively, are permanently induced. Through manipulation of the social structure of colonies, foragers can be forced to revert to nest tasks, and in the current study we investigate how protein profiles respond to such reverse development. By using a quantitative LC-MS/MS-based approach in conjunction with robust statistical validation we show that after reversal from foraging to nest activities, subsets of proteins are detected at relative concentrations that characterize nest bees, whereas other proteins remain unchanged at relative concentrations normally found in foragers. In all, we quantified the levels of 81 proteins, and for 22 of these we found significant differences between worker groups before and after reversion. We interpret these patterns as examples of plasticity and robustness at the proteome level that are linked to characteristics of behavior and aging in *Apis mellifera*.

**Keywords** *Apis mellifera* · Proteomics · Protein quantification · Behavioral reversion · Aging

## Introduction

The honey bee has long been the premier insect system for research on genetic, physiological, and evolutionary mechanisms that can underlie advanced social behavior (reviewed by Page et al. [1]). The recent availability of the honey bee genome sequence, in particular, also has made the bee amenable to modern functional genomic approaches, including proteomics [2]. Currently, the fusion of established social behavioral paradigms and new methodological tools for honey bees creates considerable potential for novel insights [3]. As a step toward such progress, we recently documented differences in the whole-body protein profiles of the major temporal caste-groups of honey bees: the nest workers and the foragers [4]. These two female castes are physiologically and morphologically distinct from queen bees (dominant reproductive females), and in addition they are characterized by specific social behavior [5]. Nest workers perform tasks inside the colony such as cleaning cells and feeding larvae, while foragers collect nectar and pollen in the field. After adult emergence, a worker bee typically performs nest-tasks for 2–3 weeks before she initiates foraging flights. A previous comparative study based on analysis of whole-body lysates (see [1] for a rationale on this global approach) revealed that the behavioral shift is linked to changes in the worker protein

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profile overall, and proposed that physiological specialization for intense flight was one possible explanation [1].

Building on these insights, we took advantage of an established plasticity in honey bee behavior to investigate the dynamic properties of the nest bee proteomes and forager proteomes. Through removal of the nest bee population of colonies, foragers were forced to return to tasks typically performed by nest bees [6]. This manipulation allowed us to determine whether whole-body protein levels were best explained by a bee's behavioral role or, rather, by the chronological progression of her life-history (her age). Previous studies on reversion of honey bee ontogeny have focused on dynamic changes in behavior, immunity, hormone levels, and transcript levels [6, 7], but associated changes in global protein profiles have not been reported so far. We demonstrate that reversion from foraging to nest tasks is linked to proteomic changes, and that reverted worker bees have hybrid characters, i.e., profiles that contain proteomic features of both nest bees and foragers.

## Experimental

### Chemicals

Unless indicated otherwise, chemicals were obtained from Sigma–Aldrich (St. Louis, USA) and Roche (Indianapolis, USA).

### Bee sampling

Workers of known age were obtained by marking newly emerged adults (<1-day-old, wild type bees) on the thorax with a spot of paint (Testors Enamel). Marked bees were reintroduced into their parental colonies in the apiary of Arizona State University (two colonies were used). A subset of marked bees that initiated foraging prior to the reversion received a second mark on their abdomen to confirm their forager status as well as their age. Baseline samples of nest workers and foragers were collected from the two hives after 25 days. Thereafter, the reversion manipulation was conducted as described before [6]. In short, all nest bees were removed from the two colonies by placing each queen in a nest box with empty wax bombs. The box with her original colony was rotated 180° and positioned on top of the new unit so the nest entrance was facing the opposite direction. Experienced foragers that subsequently were returning from flight homed to the original location of the nest entry way, thereby leading them to the unit with the queen. Naïve bees did not show this learned behavior and thus they rather returned to the old nest on top. After peak foraging hours, the top unit was removed. By then the remaining queen-containing box consisted almost entirely of experienced foragers. Under this social condition, a proportion of the foraging population will revert to nurse tasks [6].

The following bees were sampled from these experimental setups: (1) bees of known social role and unknown age ($n$=6 per group): N nest bees before reversion, F foragers before reversion, N_rev nest bees after reversion, F_rev foragers after reversion; (2) bees of known social role and known age: N_sm single-marked nest bees ($n$=6), F_sm single-marked forager ($n$=2), N_dm double-marked nest bee ($n$=5), F_dm double-marked forager ($n$=2). Methods-wise, the double-marked bees differed from single-marked bees; the second mark identified that they foraged at least 4 days prior to the reversion. Single- and double-marked reverted bees (prior foragers), single- and double-marked continuing foragers, and unmarked sisters (reverted bees and continuing foragers) were collected 11 days after the manipulation, as a previous study by our group indicates that reversion of forager physiology takes at least 8 days [6]. We used established behavioral assays to distinguish nest bees from foragers [8]. Collected bees were frozen in liquid nitrogen and stored at −80 °C until further processing.

### Protein extraction

A protein extraction mixture containing 50 mM tris pH 7.5, 6 M urea, 40% sucrose, and 1% β-mercaptoethanol (300 μL/bee) as well as tris buffered phenol (900 μL/bee) was added and individual bees were ground in this mixture for 3 min at room temperature followed by incubation at 4 °C for 20 min on a sample rotator. Subsequently, samples were centrifuged at 13,400 rcf for 5 min, and 300 μL of the upper phase were precipitated with ice-cold acetone at −20 °C overnight. On the following day the samples were centrifuged for 5 min at 9,300 rcf and the pellet was washed twice with 300 μL ice-cold methanol each. Drying was performed at room temperature and <20% relative humidity for 20 min and produced pellets suitable for further sample processing.

### Protein digestion and sample preparation for HPLC

For tryptic digestion, protein pellets were first redissolved in 50 μL of a dissolvation buffer containing 50 mM ammonium bicarbonate, 8 M urea, and 1 mM $CaCl_2$. Then, 150 μL of digestion buffer (50 mM ammonium bicarbonate, 1 mM $CaCl_2$) was added and protein concentrations were determined by Bradford using BSA as a standard [9]. For protein digestion, 10 μg protein was mixed with a

0.5 μg/μL trypsin solution (trypsin in 1 mM HCl, 1:20 ratio trypsin to sample) and proteins were incubated overnight at 37 °C. Samples were desalted on the next day using C18 extraction discs (3 M empore, St. Paul, USA) according to [10]. Desalted peptides were dried in a speed vacuum device and stored at −20 °C until further use.

### HPLC and mass spectrometry

Dried peptides (from 10 μg protein) were redissolved in 8 μL of 5% acetonitrile, 2% TFA of which 6 μL was used for analyses. Peptides were separated on a monolithic column (100-μm ID, Merck, Darmstadt, Germany) using a 105-min gradient ranging from 95% A (0.1% formic acid, 99.9% $H_2O$) to 80% B (0.1% formic acid, 99.9% acetonitrile) followed by a 15-min equilibration step. Peptides were eluted from the reversed-phase μLC column directly into an LTQ mass spectrometer (Thermo, San Diego, USA). The isolation window was set to 3 *m*/*z*, collision energy to 35, and the activation time to 30 ms. $MS^2$ was triggered for the three most abundant peaks in each MS spectrum. Using the open source search tool OMSSA [11] the spectra were matched against an *A. mellifera* sequence database retrieved from ncbi (http://www.ncbi.nlm.nih.gov/) containing additional trypsin and keratin sequences. The following criteria were used: 0.8-Da fragment tolerance, 2.0-Da precursor tolerance [12], maximum of two missed cleavages, only tryptic sequence allowed, only one hit per peptide reported, variable modifications: methionine oxidation.

Using a decoy database [13] false positive spectra were determined to make up less than 0.5% at an e-value of 0.1, which was used in all analyses. In addition, a protein hit was only accepted if the multiplication of the unique peptide score multiplied by the spectrum count gave a value ≥3. Using these additional criteria no false positive protein hits were identified in the reverse database search. Proteins were quantified using spectrum count essentially as described before [14, 15]. Data normalization was achieved by dividing the spectrum count for every protein by the value for a protein that exhibited 30% of variation over all runs and alternatively by dividing it through total spectrum count for the individual sample (see Electronic supplementary material, Table S2).

### Technical replicates

In order to test the technical reproducibility of the method one lysate produced from a nest bee was divided into three equal parts and the three samples were processed in the same manner (precipitation, digestion, LC-MS/MS, database search, quantification by spectral count). As a specific test we used the *F* test for equality of variances with the rationale that the technical error is expected to be smaller than the variance that is attributed to groups. For this procedure a standard deviation for the technical replicates was calculated for each of the proteins that varied between nest bees and foragers before the reversion. Then, the standard variation including group variance was calculated by determining the standard variance for the combined group of nest bees and foragers before the reversion. This procedure verified that the reported biological differences for these groups were on average significantly higher than the differences for the technical replicates (degrees of freedom 3, 12; *p* value 0.001).

### Statistics

Data were prepared for analysis by subtracting the median and dividing the resulting values by the standard deviation. We used non-parametric Kruskal–Wallis ANOVA to test for overall differences in protein levels and Mann–Whitney *U* tests for post hoc comparisons between the worker groups (Statistica 6.0). Type 1 error inflation was controlled by bootstrap correction of the Kruskal–Wallis alpha level (MatLab 6.5): 1,000 bootstrap iterations were run for each protein. During one iteration, six expression values were randomly assigned to each of six groups, and a *p* value calculated using the Kruskal–Wallis test. The 1,000 p values were sorted in ascending order, and the bootstrap cutoff value of the 5% lower tail was determined for the *p* value distribution. This cutoff was >0.07 for all examined proteins regardless of normalization method. Thus, our reports of significance at an alpha level of 0.05 are not associated with inflation of type 1 error. Hierarchical cluster analysis based on Pearson correlation and complete linkage clustering was used to visualize sample groups with common features (TIGR Multiexperiment Viewer version 4.0 b [2]).

To determine pattern reliability, we performed a consistency check between our current data and data from a previous study conducted with a similar methodological setup [4]. To this end, data from nest bees and foragers were used ($n$=6 in each study). Within dataset, individual protein profiles including SOD (superoxide dismutase, XP_392913), MDH (malate dehydrogenase, XP_392478), and cytochrome c (XP_391823) were normalized to the total spectrum count and sorted in ascending order relative to the values for α-glucosidase (NP_001035326). α-Glucosidase was chosen to rank profiles because it was significantly different between nest bees and foragers in both studies (higher in foragers, $p<0.05$). This result is consistent with findings from other research groups, and can be explained by the fact that the hypopharyngeal glands switch

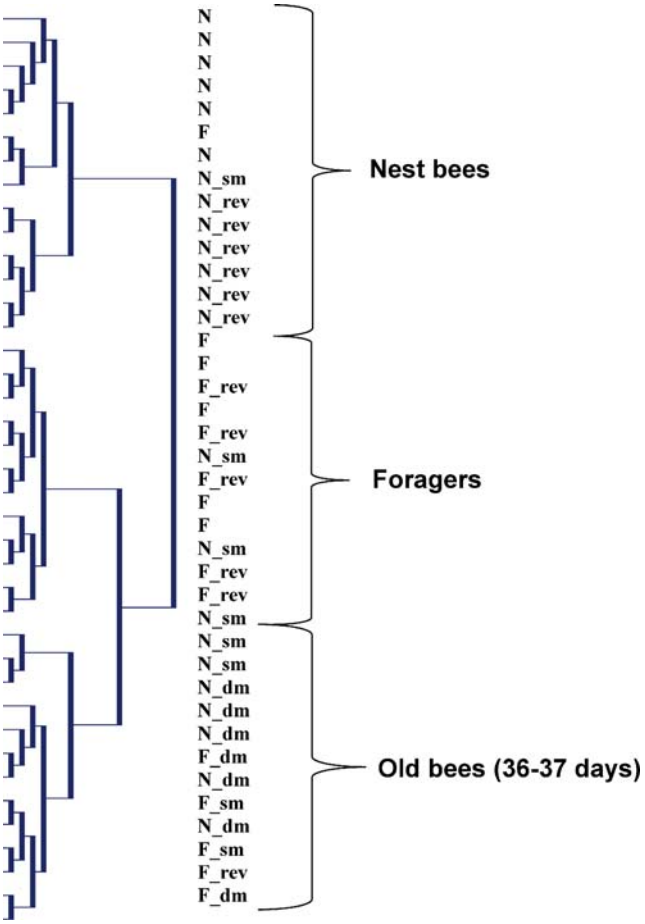


**Fig. 1** Hierarchical cluster analysis computed on the proteins that were differentially expressed among groups (22 proteins, see Electronic supplementary material, Table S2). See methods section for identification of the samples

function from producing major royal jelly protein to synthesizing α-glucosidase when workers shift from nursing to foraging activities (e.g., [16]). Cytochrome c was used as negative control (no consistent association to protein profiles was expected because patterns of cytochrome c expression diverged strongly between studies). Spearman correlation coefficients and $p$ values were calculated for the resulting data matrix (Statistica 6.0).

## Results and discussion

### Hierarchical cluster analysis reveals distinct groups

Hierarchical cluster analysis revealed three distinct clusters that separated: (i) baseline nest bees (collected prior to the reversion) and reverted unmarked sisters from (ii) foragers, and separated (iii) marked bees collected after the reversion (36- to 37-day-olds) from the two other groups (Fig. 1, see Electronic supplementary material, Table S1 for the 81 proteins that could be quantified). This divergence of nest bees from foragers was not surprising given our previous results on the differences between the whole-body protein profiles of the two behavioral groups [4]. The clustering of unmarked reverted sisters with the baseline nest bees, and the cluster of marked bees separate from others, also makes sense based on previous results. Specifically, it was shown before that the probability of reverting from foraging to nest tasks in response to social manipulation is a rapidly declining function of the number of days spent foraging [17]. This loss of plasticity was attributed to irreversible physiological fixation of forager characters and/or aging. In accord, the marked bees in our study (36- to 37-day-olds) were likely both chronologically older and had more foraging experience than the average unmarked bee (i.e., in a parallel setup with two sister colonies, age at foraging onset was 20.9±0.3 days ($n$=439) and the average forager survived for less than one week; Nilsen, Ihle, and Amdam, unpublished data). Our groups of marked foragers were thereby expected to largely reflect an irreversible and/or

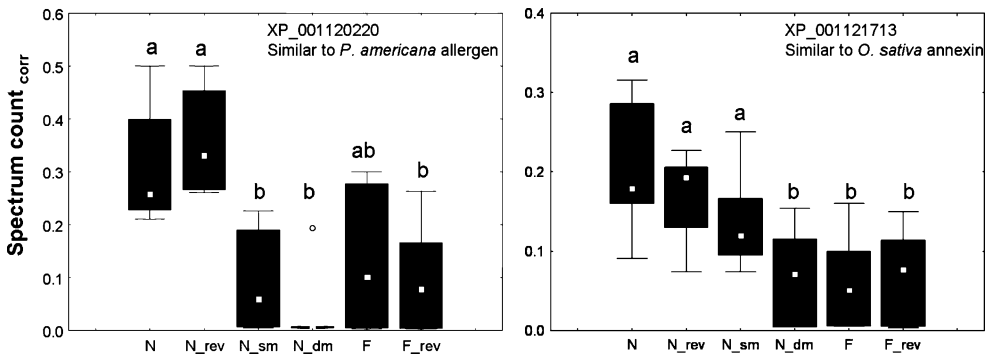


**Fig. 2** Box-and-whisker plots of proteins putatively influenced by behavior. *Boxes* represent 25–75% percentiles of the data; outliers are marked as *open circles*. Different *lowercase letters* denote significant differences between groups ($p$<0.05). For exact $p$ values, see Electronic supplementary material, Tables S1 and S2. *Spectrum count*$_{corr}$ spectrum count normalized to protein XP_625294; *left* XP_001120220, similar to *P. americana* allergen; *right* XP_001121713, similar to *O. sativa* annexin

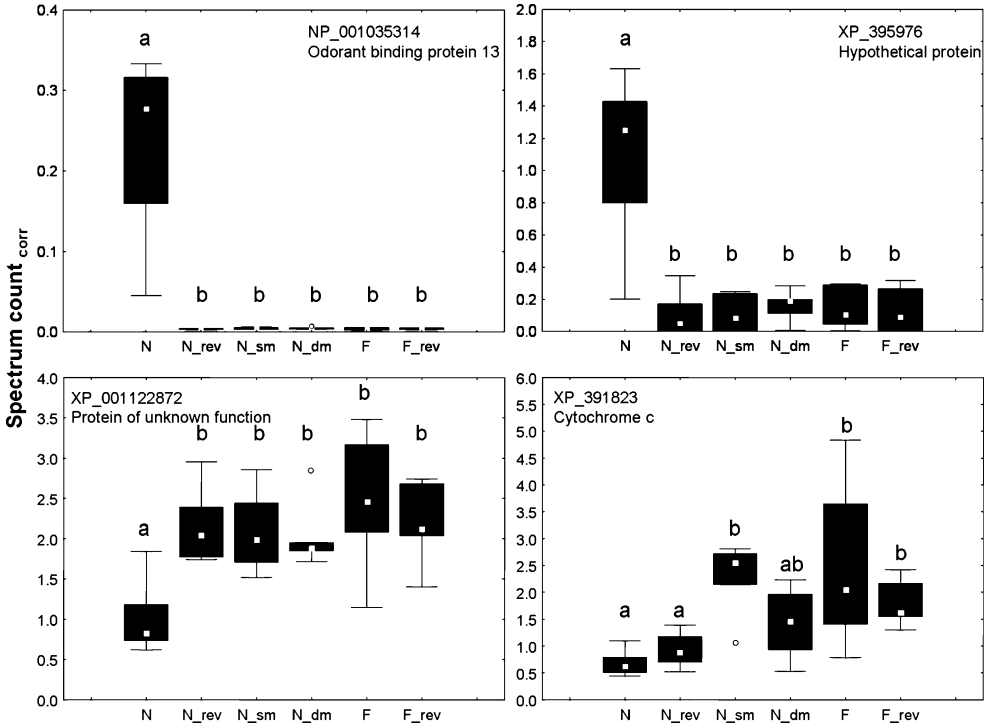


**Fig. 3** Box-and-whisker plots of selected proteins with levels that changed with age. *Boxes* represent 25–75% percentiles of the data; outliers are marked as *open circles*. Different *lower-case letters* in figures denote significant differences between groups ($p<0.05$). For exact $p$ values, see Electronic supplementary material, Tables S1 and S2. *Spectrum count*$_{corr}$ spectrum count normalized to protein XP_625294; *top left* odorant binding protein 13; *top right* hypothetical protein XP_395976; *bottom left* protein XP_001122872 of unknown function; *bottom right* Cytochrome c

senesced physiology compared to their (on average) younger unmarked sisters [17].

## Protein profiles associated with behavior

As outlined above, reverted nest bees formed a well-defined cluster closely related to the baseline sample of nest bees sampled prior to reversion (Fig. 1). These data indicate that features of the worker protein profile are coupled to behavior (Fig. 2 and Electronic supplementary material, Tables S1 and S2). In particular, two proteins putatively involved in lipid and cholesterol metabolism, two α-glucosidases, and one protein similar to a malate dehydrogenase were robustly associated with nest tasks (Fig. 2 and Electronic supplementary material, Tables S1 and S2). Our data suggest that these products exemplify proteins whose levels can change dynamically with social behavior. However, the majority of the marked (old) workers did not show this protein profile. With few exceptions they instead resembled foragers (Fig. 2 and Electronic supplementary material, Table S1). This insight supports the idea that, with time (or age), forager characters become robustly fixed and are no longer amendable to change [17]. Pooling the two sample sets of foragers of known age (F_sm and F_dm) also produced protein profiles highly similar to the patterns observed for F_rev (data not shown). Only for protein XP_001121713 (similar to *O. sativa* annexin, Fig. 2) was the median considerably higher (0.15 vs. 0.08), placing it in the range of N_sm values.

## Protein profiles associated with age

A protein with a high similarity to odorant binding proteins (OBP) showed considerable upregulation in nest bees collected before the reversion (baseline) compared to the other groups under investigation (Fig. 3, protein NP_001035314). This protein, and another product that exhibited a similar pattern (Fig. 3, protein XP_395976), are most probably associated with chronological age rather than behavior, since the baseline represents the youngest bees in our sample set (see Experimental). OBPs are small, soluble proteins (about 15 kDa) that bind other more hydrophobic substances often associated with odor recognition [18].

**Table 1** Consistency of protein profiles

| S2 \ S1 | α-glucosidase | SOD | MDH | Cytochrome c |
|---|---|---|---|---|
| α-glucosidase | 0.98 | 0.49 | 0.34 | 0.06 |
| SOD | 0.48 | 0.57 | 0.17 | 0.17 |
| MDH | 0.61 | 0.04 | 0.64 | -0.02 |
| Cytochrome c | 0.55 | 0.78 | -0.06 | -0.03 |

The matrix represents a comparison between data from a previous experiment by our group (*S1*) and the current study (*S2*). See Statistics for details. Significant positive correlation coefficients ($p<0.05$, *orange background*) denote robust protein profiles, i.e., associations that run across several proteins both within and between the two studies (*grey background* denotes $p>0.05$). The proteins are SOD (superoxide dismutase, XP_392913), MDH (malate dehydrogenase, XP_392478), cytochrome c (XP_391823), and α-glucosidase (NP_001035326)

Recently, Foret and Maleszka conducted a study including the expression pattern of *A. mellifera* OBP-mRNA levels [2]. They found expression of OBP-13 (equivalent to the one we identified on the protein level) to be highest in late larval and early pupal stages. Our findings indicate that some of the protein survives the pupal to adult conversion and is then degraded further as a function of age.

The levels of other proteins were lower in the baseline nest bees compared to the subsequently older foragers and reverted nest worker (Fig. 3, protein XP_001122872). Alternatively, they show a significantly lower value in baseline nest bees compared to foragers, and subsequently a tendency toward low values in the nest bees after reversion as in the case of cytochrome c (Fig. 3, protein XP_391823). Cytochrome c has been shown before to become more abundant with age in honey bee muscle [19]. Low values of cytochrome c after the reversion might be due to age, as indicated for some proteins in a recent study by Tofilski et al. [20].

## Concluding remarks

Here, we show that in honey bee workers the expression of some proteins is dynamically linked to behavior, while others change in relation to age. The data point to age-dependent changes in an individual's plasticity for task performance, but additional studies are required to specifically address this hypothesis. Our results are consistent with data previously obtained by our group on general differences between the whole-body proteome of nest bees and foragers [4], although protein levels are not in one-to-one correspondence between the studies (Table 1). Comparable between-setup variation is common with the techniques used here as it is for techniques used in many fields of research. Overall, our findings support the view that variations in social behaviors, which are enforced by colony needs, are reflected by the general biochemistry of the individual.

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## References

1. Page RE, Scheiner R, Erber J, Amdam GV (2006) Curr Top Dev Biol 74:253–286
2. Weinstock GM, Robinson GE, Gibbs RA, Weinstock GM, Weinstock GM, Robinson GE, Worley KC, Evans JD, Maleszka R, Robertson HM, Weaver DB, Beye M, Bork P, Elsik CG, Evans JD, Hartfelder K, Hunt GJ, Robertson HM, Robinson GE, Maleszka R, Weinstock GM, Worley KC, Zdobnov EM, Hartfelder K, Amdam GV, Bitondi MMG, Collins AM, Cristino AS, Evans JD, Lattorff HMG, Lobo CH, Moritz RFA, Nunes FMF, Page RE, Simoes ZLP, Wheeler D, Carninci P, Fukuda S, Hayashizaki Y, Kai C, Kawai J, Sakazume N, Sasaki D, Tagami M, Maleszka R, Amdam GV, Albert S, Baggerman G, Beggs KT, Bloch G, Cazzamali G, Cohen M, Drapeau MD, Eisenhardt D, Emore C, Ewing MA, Fahrbach SE, Foret S, Grimmelikhuijzen CJP, Hauser F, Hummon AB, Hunt GJ, Huybrechts J, Jones AK, Kadowaki T, Kaplan N, Kucharski R, Leboulle G, Linial M, Littleton JT, Mercer AR, Page RE, Robertson HM, Robinson GE, Richmond TA, Rodriguez-Zas SL, Rubin EB, Sattelle DB, Schlipalius D, Schoofs L, Shemesh Y, Sweedler JV, Velarde R, Verleyen P, Vierstraete E, Williamson MR, Beye M, Ament SA, Brown SJ, Corona M, Dearden PK, Dunn WA, Elekonich MM, Elsik CG, Foret S, Fujiyuki T, Gattermeier I, Gempe T, Hasselmann M, Kadowaki T et al (2006) Nature 443:931–949
3. Amdam GV, Seehuus SC (2006) Adv Cancer Res 95:31–60
4. Wolschin F, Amdam GV (2007) Proteome Sci 5
5. Winston ML (1987) The biology of the honey bee. Harvard University Press, Cambridge MA
6. Amdam GV, Aase AL, Seehuus SC, Kim Fondrk M, Norberg K, Hartfelder K (2005) Exp Gerontol 40:939–947
7. Wagener-Hulme C, Kuehn JC, Schulz DJ, Robinson GE (1999) J Comp Physiol [A] 184:471–479
8. Huang Z-Y, Robinson GE (1996) Behav Ecol Sociobiol 39:147–158
9. Bradford MM (1976) Anal Biochem 72:248–254
10. Rappsilber J, Ishihama Y, Mann M (2003) Anal Chem 75:663–670
11. Geer LY, Markey SP, Kowalak JA, Wagner L, Xu M, Maynard DM, Yang X, Shi W, Bryant SH (2004) J Proteome Res 3:958–964
12. Elias JE, Haas W, Faherty BK, Gygi SP (2005) Nat Methods 2:667–675
13. Cabelof DC, Raffoul JJ, Yanamadala S, Guo ZM, Heydari AR (2002) Carcinogenesis 23:1419–1425
14. Liu H, Sadygov RG, Yates JR 3rd (2004) Anal Chem 76:4193–4201
15. Wienkoop S, Larrainzar E, Niemann M, Gonzalez EM, Lehmann U, Weckwerth W (2006) J Sep Sci 29:2793–2801
16. Ohashi K, Natori S, Kubo T (1997) Eur J Biochem 249:797–802
17. Robinson GE, Page RE, Strambi C, Strambi A (1992) Ethology 90:336–348
18. Pelosi P, Zhou JJ, Ban LP, Calvello M (2006) Cell Mol Life Sci 63:1658–1676
19. Herold RC, Borei H (1963) Dev Biol 53:67–79
20. Tofilski A (2000) Acta Neurobiol Exp 60:35–39