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Potential costs of bacterial infection on storage protein gene expression and reproduction in queenless *Apis mellifera* worker bees on distinct dietary regimes

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ABSTRACT

Insects are able to combat infection by initiating an efficient immune response that involves synthesizing antimicrobial peptides and a range of other defense molecules. These responses may be costly to the organism, resulting in it exploiting endogenous resources to maintain homeostasis or support defense to the detriment of other physiological needs. We used queenless worker bees on distinct dietary regimes that may alter hemolymph protein storage and ovary activation to investigate the physiological costs of infection with *Serratia marcescens*. The expression of the genes encoding the storage proteins vitellogenin and hexamerin 70a, the vitellogenin receptor, and vasa (which has a putative role in reproduction), was impaired in the infected bees. This impairment was mainly evident in the bees fed beebread, which caused significantly higher expression of these genes than did royal jelly or syrup, and this was confirmed at the vitellogenin and hexamerin 70a protein levels. Beebread was also the only diet that promoted ovary activation in the queenless bees, but this activation was significantly impaired by the infection. The expression of the genes encoding the storage proteins apolipophorins-I and -III and the lipophorin receptor was not altered by infection regardless the diet provided to the bees. Similarly, the storage of apolipophorin-I in the hemolymph was only slightly impaired by the infection, independently of the supplied diet. Taken together these results indicate that, infection demands a physiological cost from the transcription of specific protein storage-related genes and from the reproductive capacity.

1. Introduction

The defense response to infection in insets is in part mediated by the hemocytes. This cellular response includes phagocytosis, hemocyte aggregation around the invader (nodulation), and formation of a multicellular capsule involving the invader (encapsulation). The cellular response is often accompanied by a humoral response which relies on enzyme cascades for hemolymph coagulation, activation of the phenoloxidase system in hemolymph leading to melanization and production of cytotoxic reactive oxygen species and reactive nitrogen species. In addition, several antibacterial peptides induced by infection in the hemocytes and fat body are secreted into the hemolymph (as reviewed by Gillespie et al., 1997; Marmaras and Lampropoulou, 2009).

The limitations of the immune response due to its physiological cost have been described in insects; indeed, mobilizing available resources to combat infection often comes at the expense of other needs (Schmid-Hempel, 2005). For example, *Drosophila* females exposed to dead bacteria lay fewer eggs, presumably because resources for egg production are redirected to synthesizing defense molecules (Zerofsky et al., 2005). Bacterially infected honey bees show a drastic reduction in the abundance of the storage proteins vitellogenin (Vg) and hexamerin 70a (Hex 70a) in the hemolymph (Lourenço et al., 2009). In this context, dietary restriction and the consequent lack of available endogenous resources have been shown to cause reduced immune reactivity in *Rhodnius prolixus* (Feder et al., 1997), *Tenebrio molitor* (Siva-Jothy and Thompson, 2002) and tsetse flies (Kubi et al., 2006; Akoda et al., 2009).

Our research interest has been focused on whether and how much the nutritionally dependent processes of protein storage and reproduction are affected by infection in the honey bee. Insect storage proteins are synthesized in the fat body and secreted into the hemolymph, where they accumulate in large quantities. These proteins are known as vitellogenin (Vg) (Wyatt, 1999; Raikhel et al., 2005), hexamerins (Hex) (Telfer and Kunkel, 1991) and

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lipophorins (Lp) (Soulages and Wells, 1994). Vg, the yolk vitellin precursor, is the major protein in the hemolymph of adult honey bee queens. It is continuously sequestered by the growing oocytes and incorporated into the yolk during vitellogenesis (Engels et al., 1990), thus serving as a nutrient reserve for the eggs and embryos. Except for the workers from the *capensis* subspecies, which regularly produce diploid female offspring without mating (throughout thelytokous parthenogenesis, Anderson, 1963), even in the presence of the queen (Moritz et al., 1999; Beekman et al., 2002), and for a described anarchistic mutant phenotype (Montague and Oldroyd, 1998), worker reproduction is low in *Apis mellifera* queenright colonies (Pirk et al., 2004) where most workers do not reproduce (Visscher, 1989). Nevertheless, a proportion of them can have functional ovaries and lay haploid male eggs (throughout arrhenotokous parthenogenesis) if separated from the queen (Jay, 1968; Visscher, 1996). Like queens, the worker bees also accumulate Vg in their hemolymph, although at lower levels. Ovary activation in workers entail increased Vg synthesis for incorporation in the growing oocyte (Engels et al., 1990; Hartfelder and Engels, 1998).

In addition to its essential function in reproduction, Vg has other important physiological roles in the honey bee. It is a zinc carrier protein that is important for hemocyte integrity (Amdam et al., 2004), it regulates the endocrine systems via regulating the juvenile hormone titer (Guidugli et al., 2005), it protects the honey bee against oxidative stress (Seehuus et al., 2006), it is involved in worker longevity (Nelson et al., 2007) and pollen or nectar foraging choice (Ihle et al., 2010).

Hexamerins are primarily storage proteins in the insect larvae hemolymph, where they constitute a source of amino acids and energy for metamorphosis (Telfer and Kunkel, 1991). The role of one of the honey bee hexamerins, Hex 70a, extends beyond its participation in metamorphosis as a storage protein because transcripts as well as the protein have been detected in developing ovaries and testes, thus suggesting a role in gonad maturation. Unlike most hexamerins that progressively disappear from the hemolymph after metamorphosis, Hex 70a persists in adult honey bee workers. Furthermore, its levels positively correlate with ovary activation in queenless workers, thus suggesting a function in reproduction (Martins et al., 2008, 2011). Circumstantial evidence that some hexamerins are targeted for egg production has also been obtained in lepidopteran and dipteran species (Benes et al., 1990; Seo et al., 1998; Capurro et al., 2000; Wheeler et al., 2000; Pan and Telfer, 2001).

In insects, a single large Lp (ApoLp-II/I) is the precursor to the ApoLp-II and -I subunits and is processed by post-translational cleavage (as reviewed in Rodenburg and Van der Horst, 2005). These subunits combine to form a high-density Lp (HDLp) that carries lipophilic compounds in the hemolymph. Another Lp, ApoLp-III, is generally found as a lipid-free molecule in the hemolymph. During times of high energy demand, however, it undergoes a conformational change and combines with HDLp to form a low-density Lp (LDLp) for transporting large quantities of lipids (Weers and Ryan, 2006). The role of Lp in reproduction has been demonstrated in lepidopteran and dipteran species, in which Lp is responsible for transporting lipids from the fat body to the growing oocyte (Kawooya et al., 1988; Sun et al., 2000). Lp has also been found in the eggs of several insects (Liu and Ryan, 1991; Telfer et al., 1991; Yun et al., 1994; Engelmann and Mala, 2005; Guidugli-Lazzarini et al., 2008).

Storage proteins titers are generally sensitive to nutritional influences. The accumulation of Vg (Bitondi and Simões, 1996) and Hex 70a (Martins et al., 2008) in the hemolymph of adult honey bee workers depends on how much pollen they consume. An absence, or even a paucity, of pollen (a protein-rich nutrient) in the diet impairs increases in both protein titers. It has also been demonstrated that feeding on high- or low-pollen diets positively correlates with high or low levels of ovary activation, respectively, in queenless honey bee workers (Hoover et al., 2006). Similarly, Human et al. (2007) showed that nourishment on protein-rich diets stimulates ovarian activation and egg development in honey bee workers. Taken together, these data establishes links between nutrition, storage protein levels and ovary activation. Indeed, in insects in general, storage protein accumulation may serve to meet the structural and energy needs of oogenesis (Wheeler and Buck, 1996; Pan and Telfer, 2001) and is dependent on food intake (Wheeler, 1996). Exceptions aside, the honey bee workers generally do not reproduce in the presence of a fertile queen. Then, why do they store proteins? Storage proteins could provide amino acids for sustaining worker basal metabolism during foraging, since foragers preferably eat nectar (Crailsheim et al., 1992), which is composed primarily by carbohydrates (Slansky and Scriber, 1985). Consistent with this hypothesis, the hemolymph titers of Hex 70a (Martins et al., 2008) and vitellogenin (Engels et al., 1990; Hartfelder and Engels, 1998), as well as the total hemolymph protein titer (Crailsheim, 1986), decrease gradually in foragers. However, the destination of proteins stored in worker hemolymph seems dependent on the social context. In case there is queen loss, workers protein reserves would then be directed to meet reproduction demands. It would not be by chance that workers accumulate storage proteins when they are younger and more prone to activate their ovaries if separated from the queen.

We hypothesized that infection affects the nutrition-dependent processes of storage of proteins and ovary activation in the honey bee. To test this hypothesis, queenless worker bees fed on diets that favors, or not, the storage of proteins and ovary activation were infected with *Serratia marcescens*. The abundance of storage protein transcripts and/or protein subunits was then investigated, as well as the ovary status (activated or non-activated). As the proteins stored in hemolymph may also be redirected to the fat body, via receptor-mediated endocytosis, to cover the costs of the defense responses, we also assessed the transcription of the genes encoding the Vg and Lp receptors (Guidugli-Lazzarini et al., 2008). In addition, we verified expression of a germ-line marker, the *vasa* gene, which is also expressed in the fat body, where it may be linked to reproduction (Tanaka and Hartfelder, 2009).

This work aimed to elucidate the costs of infection on storage protein accumulation and, consequently, on reproduction in bees on different dietary regimes.

2. Materials and methods

2.1. Honey bees

Africanized *A. mellifera* were obtained from hives of the Experimental Apiary of the Department of Genetics, Faculty of Medicine in Ribeirão Preto, University of São Paulo, Brazil. For the quantifications of transcripts and comparisons of protein levels, newly emerged worker bees (0–16 h-old) were collected from a single colony and separated in 6 groups of 40 bees that were confined in 8 × 11 × 13 cm screened wooden cages, where they were maintained during 6 days under 30 °C and 80% RH. During this period these groups of bees were fed on one of the following diets: (1) a syrup prepared with 50% sugar in water, (2) 30% beebread (the pollen processed by bees and stored in the hive) mixed with the syrup, or (3) 30% fresh royal jelly in syrup. Pure water was given *ad libitum* to the control groups. For oral infection, the same diets were offered and the bees received *ad libitum* water containing *S. marcescens* (10^5 bact/ml for the first 4 days and 10^6 bact/ml for the next 2 days). The experimental and the control groups were fed with royal jelly from the same origin (same flask), or with beebread collected from a single hive. Dead bees from each cage were scored

and removed daily, and food and water were replaced. The volume of diet consumed was measured daily. After 6 days of feeding on these diets, control and infected bees were collected for RNA and hemolymph extraction. Ovary status-dependent on the supplied diet was checked in the non-infected groups fed on syrup, beebread or royal jelly. In a parallel experiment, six groups of 40 bees from three colonies (two groups per colony) were collected and separately maintained in screened wooden cages during 9 days in the same conditions of temperature and RH described above. During this period all bee groups were continuously fed with beebread collected from a single hive. To one group from each colony it was given water (control group), and the other group from the same colony (experimental group) received water containing *S. marcescens* (10^5 bact/ml). Therefore, each pair of experimental/control groups was taken from the same colony. Water (pure and contaminated) was given *ad libitum*. After 9 days the bees were dissected and their ovaries were classified as non-activated if ovarioles were slender, without growing follicles, (comparable to the stage 1 categorized by Pirk et al., 2010), or were considered activated if containing growing follicles (comparable to stages 2–4) or fully-developed follicles (comparable to stage 5).

2.2. RNA extraction

After hemolymph collection (item 2.4), total RNA was extracted from dissected abdomens (integument and adhered fat body), using TRIzol reagent (Invitrogen). Samples containing 1 μg of total RNA were treated with DNAse (Promega) and used for reverse transcription with Superscript II (Invitrogen) and Oligo $(dT)_{12-18}$ (Invitrogen). Aliquots of cDNA were subjected to quantitative (real-time) RT-PCR and semi-quantitative RT-PCR.

2.3. Quantitative and semi-quantitative RT-PCR

Gene expression levels in abdomens of bees fed different diets, infected or not with *S. marcescens*, were analyzed using the 7500 Real Time PCR System (Applied Biosystems). Amplification was carried out with a 20 μl reaction volume, containing 10 μl of SYBR® Green Master Mix 2× (Applied Biosystems), 1 μl of cDNA (diluted 10×), 7.4 μl of water and 0.8 μl (8 pmol) of each gene-specific primer. The working genes (GenBank accession numbers is underlined) and respective primer sequences were: *vg* (AJ517411) forward 5′-GCA GAA TAC ATG GAC GGT GT-3′ and reverse 5′-GAA CAG TCT TCG GAA GCT TG-3′; *vgr* (GB16571) forward: 5′-ACC TTA CGA CAT TGC CCT-3′ and reverse: 5′-TGT GAT TTT CGG TCC AAG CCC-3′; *apoLp*-II/I (GB11059) forward 5′-AGC GAA GAG GAT CGC AGA TA-3′ and reverse 5′-AAC CCT TCG TTC CTC CTT TC-3′; *apoLpr* (XP_395858.3) forward 5′-GGT CGT TCA TGT ATA TCA TCC-3′ and reverse 5′-CGG ACA AGC ACA ACT AAG-3′; *apoLp*-III (ABY82793) forward 5′-TCT GAC AAA GCT GCG AAA TC-3′ and reverse 5′-AGT TGC GGC AGT TTG AAG TT-3′; and *hex 70a* (ABQ59246) forward 5′-GCT GGT ATC TGA ATC ACG ATT-3′ and reverse 5′-CAC GAT AAT CCG GCA AAT CG-3′. The PCR conditions were 50 °C for 2 min, and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, and the temperature is 60 °C for 1 min. Quantitative measurements were normalized using the mRNA of one of the validated housekeeping genes for *A. mellifera*, *rp49* (GenBank accession number AF441189) (Lourenço et al., 2008). The primers used for amplification of this internal control were: forward 5′-CGT CAT ATG TTG CCA ACT GGT-3′ and reverse 5′-TTG AGC ACG TTC AAC AAT GG-3′. Each run was followed by a melting curve analysis to confirm the specificity of amplification and absence of primer dimers. The relative quantification of transcript levels was calculated using the Ct method as described in Lourenço et al. (2009). To check reproducibility, each SYBR green assay was done in triplicate and repeated with three independent samples.

Expression of *vasa* (GenBank accession number GB14804) was analyzed by semi-quantitative RT-PCR. Amplifications were carried out using 1 μl (10 pmol) of specific primers (forward 5′-GAG GAA AGT TGT CTG CTG G-3′ and reverse 5′-CTC GGA TAA GAA AAC GGC-3′), 1 μl of cDNA, 10 μl of Master Mix PCR (2.5×) (Eppendorf) and 12 μl of water. PCR conditions were 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final extension step at 72 °C for 7 min. As an endogenous control we used the *A. mellifera rp49* gene. Amplification conditions were 94 °C for 2 min followed by 27 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s with a final extension step at 72 °C for 7 min. The number of cycles was carefully tested to avoid saturation. The amplification products were analyzed by electrophoresis in 1% agarose gels containing ethidium bromide, and quantified using Kodak 1D Image Analysis program, version 3.6.2 (Eastman Kodak Co.).

2.4. SDS–PAGE

Hemolymph was rapidly collected using glass microcapillaries and kept at −20 °C until the use. Aliquots of 1 μl hemolymph were analyzed by SDS–PAGE. Electrophoresis was carried out at 15 mA, according to Laemmli (1970), using 7.5% polyacrylamide gels (100 × 120 × 0.9 mm). Gels were stained with 1% Coomassie Brillant Blue dissolved in a solution of glacial acetic acid, ethanol and water (1:5:5 v/v) that was also used for gel destaining.

2.5. Statistical analysis

Data on transcript quantification and the mean volumes of diet consumed per bee were analyzed using one-way ANOVA and the Holm–Sidak test for post hoc comparisons. When the assumptions of normality for ANOVA were not fulfilled, the analyses were done using the Kruskal–Wallis and Student–Neuman–Keuls test for post hoc comparison. The Chi-square test was used for the proportions of workers with activated and non-activated ovaries. Survival analysis was done by a Kaplan–Meier log-rank test with Holm–Sidak post hoc testing for multiple comparisons. Analyses were performed with Jandel SigmaStat 3.1 software (Jandel Corporation, USA).

3. Results

3.1. Effect of diet and infection on the gene expression of storage proteins and storage protein receptors

We analyzed the expression of genes encoding storage proteins (*vg*, *hex 70a*, *apoLp-III* and *apoLp-II/I*) and encoding the Vg (*vgR*) and ApoLp (*apoLpR*) receptors in *A. mellifera* workers fed different diets (beebread, royal jelly or syrup) and infected with *S. marcescens*. A remarkable feature of the expression patterns (Fig. 1A–C) is the higher levels of *vg*, *vgR* and *hex 70a* transcripts in non-infected bees fed beebread compared to the infected bees fed the same diet. These results indicate that infection significantly prevented up-regulation of *vg*, *vgR* and *hex 70a* genes in the bees fed beebread. Infection also prevented the increase in the levels of *vg* and *hex 70a* transcripts (but not of *vgR* transcripts) in syrup-fed bees, although this effect was much less obvious than that shown by beebread-fed bees (Fig. 1A–C). Bees fed on royal jelly showed low and similar levels of *vg*, *vgR* and *hex 70a* transcripts, regardless of infection (Fig. 1A–C).

In contrast to *vg*, *vgR* and *hex 70a*, neither the diet nor the infection altered the expression of the *apoLp-III* (Fig. 1D) and *apoLp-II/I* genes (Fig. 1E). Similar to *apoLp-II/I*, the expression of *apoLpR* did not change as a consequence of the infection (Fig. 1F), however,

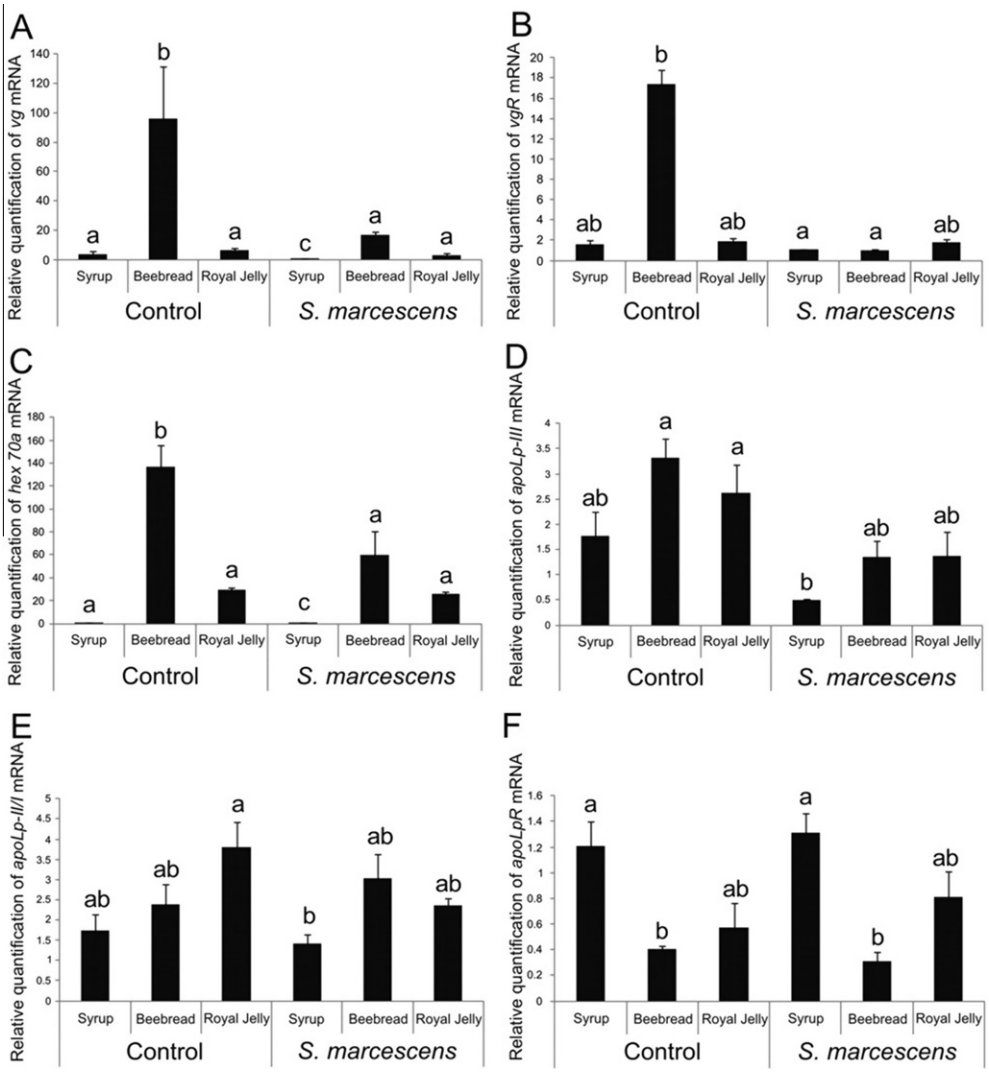

Fig. 1. Expression of genes encoding storage proteins (A) *vg*, (C) *hex 70a*, (D) *apoLp-III* and (E) *apoLp-II/I*, and storage protein receptors (B) *vgR* and (F) *apoLpR*, in the fat body of queenless worker bees fed syrup, beebread or royal jelly and infected, or not, with *S. marcescens*. Quantification of transcripts using real time RT-PCR assays. Data (means/SE of three independent samples, where each sample is representative of four bees) were normalized to *rp49* transcript levels and the relative amount of transcripts is given by $2^{-\Delta\Delta Ct}$. Different letters above bars indicate differences in transcript levels among groups (One-way ANOVA; Holm–Sidak; or Kruskall–Wallis; Student–Newman–Keuls; $p < 0.05$).

the *apoLpR* gene was the only to show a higher expression in the bees fed syrup in comparison to those fed beebread.

To validate the above findings, we investigated the abundance of *vg*, *hex 70a*, and *apoLp-II/I* gene products in hemolymph of the bees fed the different diets and infected. The Vg, Hex 70a, and ApoLp-I (the major subunit derived from the post-translational cleavage of ApoLp-II/I) proteins are secreted by the fat body into the hemolymph, where they accumulate in large quantities. Similar to the transcription levels, we observed the highest Vg and Hex 70a levels in the hemolymph of the non-infected beebread-fed bees. Intermediate and low, or very low, levels were respectively found in the other non-infected groups, fed royal jelly or syrup (Fig. 2). Infection impaired the normal accumulation of Vg and Hex 70a in the hemolymph of the bees fed beebread or royal jelly and this was more evident for Vg than for Hex 70a. Infection did not show any obvious effect on the hemolymph level of either protein in the bees fed syrup (Fig. 2). Comparisons of the ApoLp-I levels among the non-infected groups suggest that ApoLp-I accumulation is diet-dependent. However, this analysis was somewhat hindered due to the inconsistent levels of ApoLp-I in the

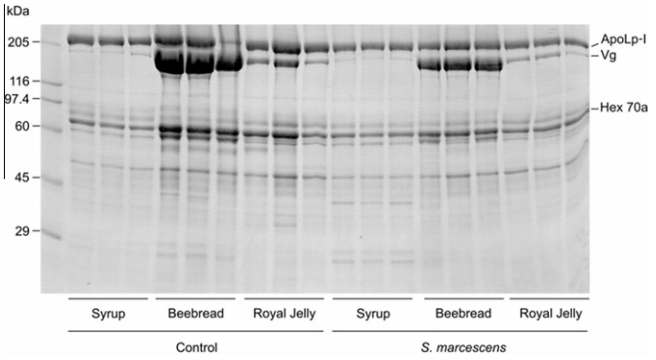

Fig. 2. Coomassie Brillant Blue-stained SDS–polyacrylamide gel showing patterns of ApoLp-I, Vg and Hex 70a proteins in the hemolymph of non-infected (control) and infected queenless bees (*S. marcescens*) fed syrup, beebread or royal jelly. Each sample corresponds to a pool of hemolymph from four bees. Molecular mass markers (kDa) are indicated at the left. (The lanes containing hemolymph of beebread-fed bees are also part of the Fig. 3C in Lourenço et al., 2009).

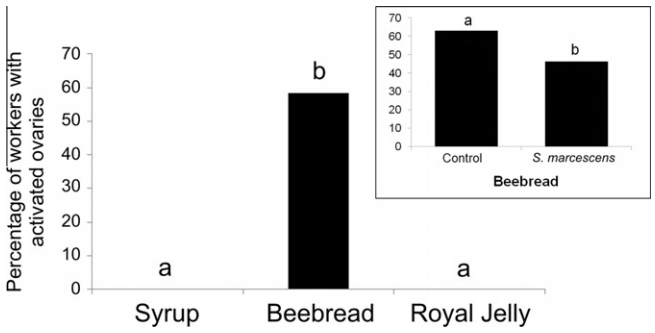

Fig. 3. Percentage of workers with activated ovaries in the groups fed syrup, beebread or royal jelly. Insert: Percentage of workers with activated ovaries in the groups fed beebread and infected with *S. marcescens*, or not infected (control). Different letters above bars indicate significant difference ($\chi^2 = 4.54$, df = 1, $p < 0.05$).

hemolymph of bees fed on each of the protein-rich diets. In any case, the infection only slightly impaired the storage of ApoLp-I, independent of the diet supplied (Fig. 2).

All the bee groups showed similar survival rates, regardless of diet or infection (Supplementary file 1). To ensure that the bees were feeding normally, we also measured the volume of food consumed daily. There was no significant difference among the groups of bees (Supplementary file 2).

3.2. Effect of diet and infection on ovary activation

We explored the relationships between diet (nutrition), ovary activation and response to infection in the honey bees. Because the worker bees used in this study were maintained without a queen, some of them were able to activate their ovaries. When we examined the bee ovaries, we found ovary activation only in the group fed beebread (Fig. 3). We then investigated whether the bacterial infection interfered with ovary activation in the beebread-fed queenless bees. Infection indeed impaired ovary activation, as was shown by a significantly lower number of bees with activated ovaries compared to the non-infected bees on this same diet (Fig. 3, insert).

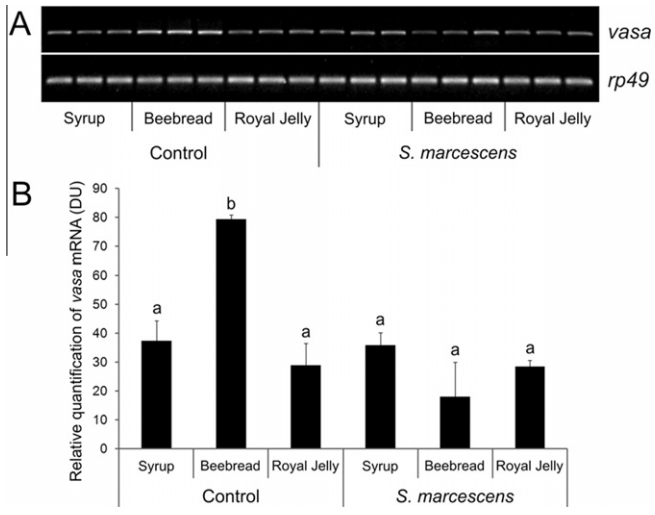

Fig. 4. Expression of *vasa* in the fat body of non-infected bees (control) and infected bees (*S. marcescens*) fed syrup, beebread or royal jelly. (A) Semi-quantitative RT-PCR followed by electrophoresis of the amplified cDNAs on ethidium bromide-stained agarose gels. The *rp49* gene was used as endogenous reference. (B) Graphs represent arbitrary densitometry units (DU) derived from the expression of *vasa* normalized to *rp49* (shown in A). Different letters indicate that transcript levels were statistically different among groups (One-way ANOVA; Holm–Sidak; $p < 0.05$).

3.3. Effect of diet and infection on expression of the vasa gene

To investigate whether the effects of nutrition and infection extended to other reproduction-related genes (in addition to storage protein and receptor genes), we analyzed the *vasa* transcripts levels in the bees fed on different diets and challenged with *S. marcescens*. Significantly higher *vasa* transcripts levels were observed in the bees fed beebread than in those fed the other diets (Fig. 4). Like observed for the *vg*, *vgR*, and *hex 70a* genes, bacterial infection impaired the increase in *vasa* transcript levels in the beebread-fed bees (Fig. 4).

4. Discussion

In the present study, we explored the costs of bacterial infection on gene transcription, protein storage and ovary activation in honey bee workers in relation to the type of the supplied diet.

In a previous study (Lourenço et al., 2009), we used injection rather than oral administration to bacterially infect bees and then analyzed *vg* and *hex 70a* expression at 12 h post-infection. The transcript and protein-level responses to bacterial injection were not distinguishable from those caused by water injection (injury). In the present work, the injury effect was circumvented by orally administering the bacteria via the diet. In addition, we extended the duration of the experiments (to 6 and 9 days) and considered additional parameters, i.e., nutrition and ovary status (activated or non-activated). Three other genes (*vgR*, *apoLpR* and *vasa*) were also investigated in the current study.

4.1. Infection altered the transcription of vg, vgR, hex 70a and vasa, and this was very evident in beebread-fed bees

Notably, the cost of infection on transcription and protein levels was mostly evident in the beebread-fed bees. In these bees, the transcription of *vg*, *vgR*, *hex 70a* and *vasa*, and the levels of Vg and Hex 70a proteins, were clearly impaired by the infection. These results indicate that the physiological cost of infection is better evidenced under certain dietary conditions. Furthermore, the dynamic process of Vg storage (in hemolymph) and mobilization (to the fat body) may have been disrupted since the expression of *vgR* was inhibited in beebread-fed bees as a consequence of the infection.

Royal jelly, like beebread, is a rich source of proteins for bees. It might be thought that the proportion of royal jelly in the diet was insufficient to allow increased levels of *vg*, *vgR*, *hex 70a* and *vasa* transcripts, and the Vg and Hex 70a proteins. Alternatively, the diet could have provided an excess of royal jelly and caused adverse effects on transcription. It is known that high levels of dietary protein consumption negatively correlate with survival in young worker honey bees (Pirk et al., 2010). This alternative, however, seems unfounded because feeding on royal jelly did not cause significant bee mortality in our study. Furthermore, the levels of *apoLp-III*, *apoLp-II/I* and *apoLpR* transcripts did not significantly differ between bees fed on royal jelly or beebread. Together, these results suggest that diet differentially regulates gene activity. The expression of *apoLpR*, which was up-regulated in bees fed syrup, reinforces this idea.

The *vasa* gene is a germline marker in the ovaries (Dearden, 2006). It is also expressed in the fat body of honey bee queens but not in queenright workers. This observation has led to the hypothesis that *vasa* may play a role in queen fertility (Tanaka and Hartfelder, 2009). In the current study, we detected *vasa* expression in the fat body of queenless worker bees. Interestingly, *vasa* expression was up-regulated in the queenless bees fed beebread, which tended to have activated ovaries. This finding

supports a possible role for this gene in fecundity. If so, through the inhibition of *vasa* expression the infection may also have affected bee fecundity.

Therefore, *S. marcescens* infection was costly to the honey bee, resulting in harmful effects on transcription, hemolymph protein storage and ovary activation. We had three main reasons to choose *S. marcescens* for the infections: (1) It is potentially pathogenic for insects (Steinhaus, 1959) and was associated to septicaemia in adult honey bees (Wille and Pinter, 1961). The isolation of *S. marcescens* from diseased honey bee larvae, followed by the reproduction of the disease experimentally, evidenced the pathogenicity of this microorganism (El-Sanousi et al., 1987), (2) as we demonstrated (Lourenço et al., 2009) *S. marcescens* was efficient in activating the honey bee immune system, (3) furthermore, and more importantly, *S. marcescens* is not lethal when the infection occurs orally, via food (see Steinhaus, 1959). Although *S. marcescens* is highly pathogenic when inoculated into the insect hemocoel, it is only mildly pathogenic when ingested (Bulla et al., 1975). This feature is very important, considering that the accumulation of proteins in hemolymph, as well as the ovary activation (in orphaned bees), occurs gradually as the bees age. Thus, we used in our experiments a non-lethal bacterium, able to activate the immune system but allowing the survival, so that the infection costs in terms of transcription and storage of hemolymph proteins, and ovary activation, could be conveniently assessed.

4.2. The expression of apoLp-III, apoLp-II/I, and apoLpR was not significantly altered by infection, regardless of diet

The infection did not appear to demand a significant cost from apolipophorins (*apoLp-III*, *apoLp-II/I*) and the apolipophorin receptor (*apoLpR*) transcriptions.

In addition to its role in lipid transport, ApoLp-III has been shown to play a role in inducing antimicrobial proteins and phagocytosis by hemocytes (Wiesner et al., 1997; Kim et al., 2004). It is known that ApoLp-III binds to bacterial surface components in *Galleria melonella*, thus playing an important role in the immune response (Halwani et al., 2000). Thus, the lack of induction of the honey bee *apoLp-III* gene expression in response to the infection is inconsistent with what would be expected if this gene is important for immune defense. However, ApoLp-III was similarly not induced in *Anopheles gambiae* after *Plasmodium falciparum* or *Plasmodium berghei* infection (Mendes et al., 2008). In a previous experiment (Lourenço et al., 2009), we observed down-regulation of *apoLp-III* expression in bees under a different, and perhaps more drastic, experimental condition, i.e., after injection with bacteria (*S. marcescens* or *Micrococcus luteus*). Under this specific condition, the cost of infection on *apoLp-III* transcription became evident. Therefore, neither of these two experimental infection conditions (oral or via injection) caused induction of *apoLp-III* expression that could be interpreted as a specific defense reaction.

The *apoLp-II/I* transcript levels were not significantly altered by diet or infection. However, the effect of the diets on ApoLp-I accumulation was not as obvious as that seen for Vg. It seems that the diets have little effect on ApoLp-I hemolymph levels, but this analysis is somewhat hindered by the diverged levels of this protein subunit among bees fed the same diet (beebread or royal jelly). The bacterial infection barely altered the hemolymph ApoLp-I storage. In addition to its roles in lipid transport, the product of the *apoLp-II/I* gene binds to lipopolysaccharides from bacterial wall (Kato et al., 1994; Ma et al., 2006). It has also been shown that the expression of this gene and of the gene encoding the apolipophorin receptor is significantly enhanced in *Aedes aegypti* after bacterial infection (Cheon et al., 2006). This important role in defense against bacteria may explain why *apoLp-II/I* transcripts and ApoLp-I subunits remain relatively abundant in infected bees. Accordingly, the transcription of the apolipophorin receptor, *apoLpR*, was also not affect by infection, suggesting that the process of mobilization of its ligand (apolipophorin) from hemolymph to the fat body was preserved.

4.3. Diet-dependent ovary activation was impaired by the infection

In general, the storage of proteins and other compounds in the hemolymph occurs under conditions of high nutrient availability. In the honey bee there is a positive correlation between nutrition and hemolymph levels of Vg (Bitondi and Simões, 1996) and hexamerins, including Hex 70a (Cunha et al., 2005; Bitondi et al., 2006; Martins et al., 2008). Nutrition has also been shown to be highly correlated with ovary activation and reproduction in the honey bee. Indeed, protein-rich diets promote ovary activation in queenless bees and even in queenright bees (Lin and Winston, 1998; Pernal and Currie, 2000; Hoover et al., 2006; Human et al., 2007; Pirk et al., 2010). Pollen is the main source of dietary proteins for bees, and may vary in composition and protein content, which influences on ovary activation and egg development (Pernal and Currie, 2000; Human et al., 2007).

In our experiments, only the queenless bees showing the higher levels of storage proteins, i.e., the beebread-fed bees, had active ovaries. This result is consistent with the diet inducing intense protein synthesis to provide resources for ovary activation. Infection significantly impaired ovary activation in the beebread-fed bees strongly suggesting that diet-derived resources were diverted away from reproduction to attend to the critical needs of infection.

Our results linking a pollen-derived diet (beebread), but not royal jelly, with ovary activation seem in contrast to previous studies (Lin and Winston, 1998; Altaye et al., 2010) showing that royal jelly promoted ovarian activation better than pollen or a pollen substitute. Furthermore, it was already considered (Schäfer et al., 2006 and references therein) that in contrast to pollen, the royal jelly is rapidly and completely digested, whereas feeding on pollen would be physiologically more costly. In our experiments, however, the caged bees were fed on fresh beebread directly collected from the hive stocks, making it difficult to compare our results with those obtained by feeding bees on pollen or pollen substitutes. Beebread is extensively manipulated by the bees and has a different composition and nutritional quality. It is made of partially digested pollen mixed with honey and enzymes, and certainly it is more easily digestible and utilizable than pollen.

The natural and basic nutrients for the young worker bees, like those used in our experiments, are pollen and honey. Pollen is consumed by these bees, which have a high digesting capacity and use pollen as raw material for jelly production in the hypopharyngeal glands. In colony conditions, the jelly is transferred via trophallaxis mainly to larvae and queens, but also to workers and drones (Crailsheim, 1992, 1998), emphasizing that the young workers are producers of royal jelly, rather than recipients (Thompson et al., 2006). The caged bees in our experiments may have directly used the products derived from pollen (beebread) digestion for ovary activation. It is also possible, however, that the digested products were also used for jelly production. Without brood to rear, the jelly may then have been transferred via trophallaxis from one caged bee to another, thus contributing as raw material and energy for ovary activation.

Ovary activation in queenless workers depends on the balance of nutrients in the diet. Even being artificial, a balanced diet may favor ovary activation (Pirk et al., 2010). By presenting queenless bees with choices between complementary diets made with varied protein to carbohydrate proportions, Altaye et al. (2010) highlighted the importance of the optimal balance of nutrients for ovary activation. The lack of ovary activation in our bees fed on royal jelly plus syrup may tentatively be ascribed to an imbalance

in the protein to carbohydrate ratio, but this requires further investigation.

It is known that the *A. mellifera* queen mates with several drones (Palmer and Oldroyd, 2000, for review), and this markedly has an effect on colony genetic structure such as the worker population is subdivided in subpopulations formed by distinct patriline genotypes. In a queenless condition, several workers activate their ovaries and become egg layers (Velthuis, 1970), but this can significantly differ between colony patrilines thus reflecting genotype constitution (Makert et al., 2006). The enhanced fertility in some patrilines may involve predisposition for a faster activation of the ovaries (Page and Robinson, 1994; Oldroyd et al., 2001; Martin et al., 2002), and additionally, may be linked to a developmental ability to maintain a higher ovariole number (Makert et al., 2006), considering that the degeneration of most of the ovarioles in worker-destined larvae, but not in queen-destined larvae is part of the caste differentiation program (Hartfelder and Steinbrück, 1997; Schmidt Capella and Hartfelder, 1998).

In our experiments, 6 groups containing each 40 newly emerged worker bees from 3 honey bee colonies (2 groups randomly collected per colony) were confined during 9 days in small cages to assess the costs of bacterial infection on ovary activation (Fig. 3, insert). Beebread was given to all group pairs to propitiate ovary activation, but only one group in each pair was bacterially infected. Considering the polyandry inherent to *A. mellifera* queen reproduction, it is very possible that the variety of intracolonial patrilines was not equally represented in the group pairs. However, neither in a standard colony the patrilines are equally present at a given time period. As in our experiments each group pair was collected from the same colony, headed by a single queen, there was a certain degree of population homogeneity. The genotypic discrepancies between the group pairs were not sufficient to obfuscate the effect of infection on ovary activation.

Altogether, our results demonstrate a relationship between nutrition and effect of infection on transcript and protein levels, and ovary status (activated/non-activated). In beebread-fed bees, the bacterial infection was costly in terms of transcription of *vg*, *vgr*, *hex70a* and *vasa* genes and storage of Vg and Hex 70a proteins. Furthermore, the costs of infection impaired ovary activation. There has been recent evidence in the literature that the genes and proteins involved in biological processes other than the production of immune effectors are down-regulated by infection (Scharlaken et al., 2007, 2008). Two putative storage protein genes were markedly repressed after bacterial infection in the eri-silkworm *Samia cynthia ricini*, suggesting that infection shuts down expression of dispensable genes in favor of immune-related genes (Meng et al., 2008). Similarly, parasitism in *Drosophila* caused reductions in the size and number of eggs (Fellowes et al., 1999), which may indicate that resources destined for reproduction are diverted to mount an immune response. Using proteomics Chan et al. (2009) observed that honey bee larvae responded to infection with *Paenibacillus larvae* by depleting their energy stores and producing proteins to directly combat the bacteria. In this case the infected larvae showed a significant reduction of hexamerins, lipid carriers, retinoid- and fatty-acid binding proteins and apolipophorin III. The honey bee larvae also showed significant reduction of *hex 70b* and *vg* transcripts when up-regulation of immune-related transcripts was triggered by fungal infection (Aronstein et al., 2010). These results, along with our findings imply that the bees face infection by diverting their energy stores towards immunity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2012.06.006.

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