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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