UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CAMPUS DE BOTUCATU

SUPLEMENTAÇÃO DE FONTE INORGÂNICA DE ZINCO NO PERFIL PROTEÔMICO E METALÔMICO DA GELEIA REAL EM ABELHAS *Apis mellifera* **L.**

AIMÊ DE ALMEIDA LONGUINI

Dissertação apresentada ao Programa de Pós-graduação em Zootecnia como parte das exigências para obtenção do título de Mestre em Zootecnia

BOTUCATU – SP

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AIME DE ALMEIDA LONGUINI

ZOOTECNISTA

Orientador: Prof. Dr. Ricardo de Oliveira Orsi

Co-orientador: Prof. Dr. Samir Moura Kadri

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BIOGRAFIA DO AUTOR

Aimê de Almeida Longuini – nascida em 8 de julho de 1992, na cidade de Santa Cruz das Palmeiras/SP, filha de João de Melo Longuini e Janaína Almeida. Ingressou no curso de Zootecnia da Universidade Estadual Paulista "Júlio de Mesquita Filho" – Unesp – Faculdade de Medicina Veterinária e Zootecnia, Câmpus de Botucatu, em março de 2014, onde foi bolsista Fapesp e Pibic e realizou três iniciações científicas e um estágio BEPE em Portugal na Faculdade de Coimbra. Em março de 2019 iniciou o curso de Mestrado em Zootecnia da Unesp – Faculdade de Medicina Veterinária e Zootecnia – Câmpus de Botucatu. Atua na área de nutrição e produção animal.

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> “Mas tu não deves esquecer. Tu te tornas eternamente responsável por aquilo que cativas.”

O pequeno príncipe - Antoine de Saint-Exupéry

RESUMO GERAL

Este estudo teve como objetivo avaliar a qualidade nutricional do perfil proteico da geleia real produzida pelas abelhas *Apis mellifera* suplementadas com diferentes concentrações de zinco inorgânico (Sulfato de Zinco monoidratado - 0, 25, 50 e 75 ppm). Foi feita eletroforese bidimensional para o fracionamento das proteínas da geleia real e o nível de zinco quantificado pela técnica de espectrometria de absorção atômica com chama (FAAS). A análise de proteínas da geléia real como componente extracelular foi realizada pela plataforma Blast2Go, onde foram obtidos gráficos de funções moleculares e processos biológicos das proteínas encontradas através da análise proteômica por análise de espectrometria de massa ESI MS / MS, mostrando as proteínas "Major Royal Jelly Protein 1" e "Major Royal Jelly Protein 8" como as principais proteínas ativas na geléia real tanto para up como para down regulation e associada ao zinco. Todos os tratamentos independentes das concentrações de zinco mostraram número menor de *Spots* proteicos quando comparadas com o controle. Todas as proteínas contendo zinco foram classificadas como Proteínas Principais da Geleia Real (MRJPs). A exposição de abelhas nutrizes ao mineral zinco em sua forma inorgânica promoveu aumento na expressão de proteínas da geleia real envolvidas em sistemas de defesa (MRJP8 e MRJP9), entretanto reduziu a expressão de seis diferentes proteínas das MRJPs (sendo elas: MRJP, MRJP1, MRJP2, MRJP3, MRJP5 e MRJP7). Nossos resultados demonstram que proteínas vitais e processos metabólicos são prejudicados em abelhas nutrizes expostas ao mineral zinco em sua forma inorgânica em todas as doses utilizadas afetando a nutrição e manutenção de colônias.

Palavra - chave: abelhas, mineral, proteômica, suplementação.

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1.0. Histórico da abelha Apis mellífera no Brasil

O contato entre abelhas e humanos é antiga e comprovado por diversos registros como manuscritos de livros científicos e pinturas rupestres da idade da pedra lascada, mostrando a colheita do mel pelo homem de maneira extrativista, o que causava grandes danos aos enxames prejudicando sua longevidade e a integridade da colmeia (Crane, 1999).

A partir do momento em que o homem teve contato com as abelhas, embora de maneira extrativista, ocorreu um marco na criação de abelhas e com o passar do tempo foram surgindo técnicas de aperfeiçoamento desta criação de maneira racional, hoje a apicultura oferece manutenção da vegetação nativa, impulsionamento da agricultura e amplas oportunidades de geração de empregos (Bhatnagar et. al., 2020).

A introdução das abelhas no Brasil começou por volta do século XIX e se resumia em duas espécies principais, *Apis mellifera mellifera* de origem alemã e *Apis mellifera ligustica* de origem italiana, estas duas espécies apresentavam baixa defensividade e produtividade quanto a pastagem apícola disponível (Gonçalves, 2001; Wiese, 2005; Faita et al., 2014).

As primeiras abelhas europeias foram introduzidas no Brasil pelo padre Antonio Carneiro Aureliano que com uma autorização do rei D. Pedro II, em março de 1839, conseguiu importar abelhas provenientes da cidade de Porto em Portugal, com o intuito na produção de cera, pois, neste período a cera era muito utilizada para confeccionar velas para uso em atividades religiosas nas igrejas, contudo, foram instaladas colmeias na cidade do Rio de Janeiro (Wiese, 2005).

Em 1845, na região do Rio Grande do Sul foram introduzidas as abelhas europeias *Apis mellifera mellifera* de origem alemã, trazidas por imigrantes alemães que deram início à apicultura nesta região. Entre os anos de 1870 e 1880 foram introduzidas as abelhas *A. mellifera ligustica* de origem italiana trazidas por imigrantes europeus que as propagaram por todo território brasileiro (Trindade et al. 2004; Wiese, 2005).

Em 1956, 49 abelhas rainhas *Apis mellifera scutellata* foram trazidas da África do Sul e Tanzânia e introduzidas em colônias testes no Brasil, na cidade de Rio Claro pelo professor Warwick Stevam Kerr com o objetivo de melhorar a produção nacional de mel (Faita et al., 2014).

Contudo, em 1957, vinte e seis enxames experimentais com suas respectivas rainhas africanas escaparam e assim iniciou o processo de africanização das abelhas, com predominância das características das abelhas africanas, apresentando comportamento higiênico, resistência a doenças, adaptação ao clima tropical, comportamento altamente defensivo e enxameatório (Winston, 1992; De Jong, 1996; Gonçalves, 2006; Faita et al., 2014;).

Em torno de quarenta anos após 1956, as abelhas africanizadas já estavam presentes em boa parte do continente sendo limitadas apenas por barreiras físicas e climáticas, a migração para lugares longínquos pode ser justificada pela capacidade desta nova abelha voar longas distâncias e sua alta capacidade de enxamear (Del lama, 2004; Mitchell, 2006).

1.1. As abelhas Apis mellifera L.

As abelhas melíferas são insetos sociais que pertencem à Família *Apidae* da Ordem *Hymenoptera* e se destacam como polinizadores devido à facilidade de manejo, generalidade e fidelidade na polinização, contribuindo diretamente para o aumento e garantia da produção agrícola (Morse e Calderone, 2000; Eyer et al., 2017; Saunders 2018).

As abelhas vivem em uma complexa sociedade com compartilhamento do ninho, cuidado da prole, sobreposição de gerações e divisão de castas (Yan et al., 2014). As castas da colmeia são representadas pela rainha, responsável pela função reprodutiva sendo a única capaz da fertilização e postura de fêmeas e machos na colônia, sendo o único membro da colmeia que recebe alimentação exclusiva de geleia real desde seu nascimento até sua morte; os zangões são responsáveis apenas pela reprodução e as abelhas operarias, responsáveis por funções que variam de acordo com a idade e desenvolvimento glandular (Crailsheim, 1992; Rueppell et al., 2016; Eyer et al., 2017).

a  b  c 

Figura 1. Representação das castas de uma colmeia: rainha (a), zangão (b) e operária (c) **Fonte:** Aimê Longuini.

Até o 3º dia após a emergência da célula de cria, as abelhas operárias desempenham função de faxineiras e realizam a limpeza da colmeia, do 4º ao 12º dia são denominadas nutrizes onde alimentam as rainhas e crias com secreções de suas glândulas, do 13º ao 18º dia de vida adulta, as abelhas são chamadas de engenheiras, pois são responsáveis pela produção de cera e construção do ninho, do 19º e 20º dia são denominadas guardiãs e desempenham função de proteger o ninho contra invasores, do 21º até sua morte a abelha desempenha função de campeira, indo em busca de recursos como água, resinas e alimento (Wiese, 2005).

2.0. Exigências nutricionais das abelhas Apis mellifera L.

Para que a colônia possa expressar todo seu potencial produtivo e reprodutivo, nutrientes específicos são necessários para proporcionar a manutenção e sobrevivência do enxame (Di Pasquale et al., 2013). Os nutrientes são coletados pelas abelhas através de recursos florais disponíveis no campo, sua alimentação é baseada em néctar como fonte de energia, pólen como fonte proteica e a água que cumpre papel de transporte e dissolução de substâncias e serve de meio para várias reações químicas (Berenbaum & Calla, 2020).

O néctar é constituído de três açucares simples a sacarose, glicose e frutose, que variam em suas quantidades de acordo com as espécies das plantas, as abelhas campeiras levam o néctar até a colmeia e entregam para as abelhas armazenadoras que irão regurgitar este néctar floral e transforma-lo em mel reduzindo significadamente o teor de água, o mel será consumido pelas abelhas em sua forma menos concentrada e apenas uma pequena parte será armazenada nos favos (Wright et. al., 2018).

Por ser feito a partir do néctar das flores o mel apresenta diferentes propriedades físicas e químicas, por isso sua produção depende da abundância e qualidade das flores que estão no raio de ação das abelhas. Portanto, o mel apresentará características diferentes, principalmente quanto à cor, sabor e perfume, dependendo de sua localização geográfica e florada assim como o pólen (Gois et al., 2013; Wright et al., 2017).

O pólen é fundamental para a dieta das abelhas, uma vez que fornece um conjunto de nutrientes como proteínas, lipídios, vitaminas e minerais que variam de acordo com as espécies vegetais, condição nutricional da planta e do ambiente durante seu

desenvolvimento (Brodschneider & Crailsheim, 2010; Wright et al., 2017). É importante que exista a variedade de espécies vegetais para que as abelhas possam balancear estes nutrientes para elaborar o "pão de abelha", que é uma mistura fermentada de pólen, mel e secreções salivares das abelhas (Wright et al., 2018).

Componente importante para a saúde individual da abelha e da colônia, os nutrientes do pólen impactam no crescimento, longevidade e imunocompetência. As abelhas operárias do seu quinto ao décimo quinto dia de vida aproximadamente consomem grandes quantidades de pólen para obter um nível adequado de proteínas e aminoácidos necessários para completar o seu crescimento, desenvolvimento e a atividade de secreção de suas glândulas hipofaringeanas também responsáveis pela produção da geleia real (Corby-Harris et. al., 2018).

A geleia real é de extrema importância para a colônia, servindo como alimento para larvas de operárias, rainhas e zangões até o terceiro dia de vida; após este período, larvas de operárias e zangões recebem pólen e néctar/mel como principal alimento e pequenas quantidades de geleia real; entretanto, a larva de rainha recebe exclusivamente geleia real durante todo seu desenvolvimento larval e vida adulta, propiciando alterações fisiológicas como desenvolvimento de estruturas relacionadas à reprodução (Ohashi et al.,1999; Evans & Wheeler, 2001).

A geleia real é formada a partir da mistura de secreções das glândulas hipofaringeanas (secreção clara e rica em proteínas) e mandibulares (componente branco leitoso), localizadas na cabeça das operárias, entre o 5º e o 15º dias de vida, sendo considerada um dos principais alimentos consumidos pelas abelhas (Coelho et al., 2008; Zheng et al., 2011; Fujita et al., 2012; Lin et al., 2018).

Abelhas operárias jovens que não consomem quantidade ideal de proteínas em sua dieta podem apresentar produção de geleia real prejudicada e a deficiência em termos nutricionais pode afetar o desenvolvimento da colônia. A composição da geleia real também pode variar dependendo da idade da larva, condições ambientais, estado fisiológico e metabólico da abelha nutriz (Albert et al., 1999; Standifer 2003; Scarselli et al., 2005; Lin et al., 2018).

A geleia real possui em sua composição proteínas hidrossolúveis (Water Soluble Proteins - WSP) que apresentam destacado valor nutricional e são denominadas de Proteínas Principais da Geleia Real (MRJPs), que representam 82% do total das proteínas hidrossolúveis e cerca de 90% do total de proteínas da geleia real, cuja massa molecular relativa é de 49 a 87 kDa (Schmitzova et al., 1998; Scarselli et al., 2005). Foram

encontradas até o momento nove proteínas das MRJPs em *Apis. mellifera* (MRJP1, MRJP2, MRJP3, MRJP4, MRJP5, MRJP6, MRJP7, MRJP8, MRJP9), e um polipeptídio incompleto, MRJPψ, codificado por um pseudogene (Drapeau et al., 2006).

As abelhas dependem de recursos naturais e devido a isso temos oscilações de produção e mantença durante os períodos de ausência de florada, período onde a reserva de alimento na colônia pode ser insuficiente, podendo assim, prejudicar o balanço de nutrientes necessários para manutenção das colônias e adequado funcionamento do metabolismo das abelhas (Herbert, 2000; Naug, 2009; Alaux et al., 2010).

Devido às reduções na disponibilidade de recursos florais no ambiente, o fornecimento de dietas artificiais é uma estratégia importante para manutenção de colônias, sendo importante o desenvolvimento de estudos para melhorar a nutrição das abelhas e reduzir as perdas de colônias (Herbert, 2000; Alaux et al., 2010; Degrandi-Hoffman et al., 2010; Morais et al., 2013; De Sousa Gomes et. al., 2019).

2.1. Zinco

Apesar dos alimentos energéticos e proteicos serem de extrema importância na alimentação das abelhas, nutrientes como vitaminas e minerais são indispensáveis para o desenvolvimento das colônias (Haydak, 1970; Eyer, 2016). De todos os minerais que se ligam a proteínas, o zinco é o mais abundante (Shi & Chance, 2011), tornando-se assim um foco de investigação nos processos de controle da expressão gênica e outros mecanismos biológicos (Vallee et al, 1993).

As fontes minerais são obtidas através do pólen, néctar e água, Herbert e Shimanhuki (1978) propõem uma dieta com 1000 ppm de potássio, 500 ppm de cálcio, 300 ppm de magnésio e 50 ppm de sódio, zinco, manganês, ferro e cobre. Zhang et al. (2015) sugerem 30 ppm de zinco para a sobrevivência e atividades antixiodantes para abelhas e 60 a 70 ppm para a produção de geleia real. Contudo, existe pouco conhecimento sobre a necessidade desses micronutrientes e suas influencias fisiológicas nas abelhas (Brodschneider e Crailsheim, 2010).

Minerais como o zinco são fundamentais como componentes estruturais e funcionais; no aspecto estrutural está presente nas metaloproteínas, e no aspecto funcional, atua como cofator enzimático, participando do metabolismo, regulação da expressão gênica, manutenção estrutural de biomembranas, imunidade e proteção contra radicais livres, síntese de proteínas, entre outras (Maret 2017; Abd El-Hack et al., 2020).

O zinco ligado às proteínas exerce funções catalíticas e inibitórias enzimáticas, arranjos e desarranjos de complexos de subunidades macromoleculares e formação de proteína/receptor complexo (Maret 2017; Abd El-Hack et al., 2020).

3.0. Proteômica e metaloproteômica

A análise proteômica permite o estudo do perfil proteico da geleia real, demonstrando efeitos estruturais e funcionais causados sob a influência de diferentes estímulos (Li et al., 2007; Kamakura, 2011; Fujita et al., 2012). O proteoma é o conjunto de proteínas expresso por um determinado organismo em um dado momento, ou seja, é um complemento proteico do genoma com intuito de esclarecer os mecanismos bioquímicos e fisiológicos ao nível molecular, permitindo visualizar alterações das proteínas quando se compara células ou condições diferentes, na prática pode ser classificada como proteômica da expressão ou estrutural (Singh et al., 2016; Huttlin et al., 2020).

Os íons metálicos estão ligados às proteínas e metaloproteínas, são essenciais como componentes estruturais com função integrante de compostos orgânicos corporais e funcionais com função catalisadora de sistemas enzimáticos (De Paula Araújo et al., 2019; Rittle et al., 2019). Para a realização da atividade biológica estima-se que por volta de 40% das proteínas e enzimas necessitam da presença de um íon metálico (Romanowski et al., 2001; Garcia et al., 2006; Blindauer, 2019). Processos catalíticos, como ligação a um substrato e ativação do mesmo, transporte e armazenamento são resultados de processos metabólicos, assim como conversão de energia na fotossíntese e respiração, expressão e regulação gênica, sendo os íons responsáveis por tais processos (Romanowski et al., 2001; Coutinho et al., 2020; Huo et al., 2020).

As metaloproteínas são caracterizadas pela alta afinidade da interação metal-proteína através de ligação especifica, assim sendo realizada uma ligação de difícil quebra, contudo proteínas de metal ligante caracterizam-se pela baixa afinidade da ligação metal-proteína através de ligações não especificas, sendo esta facilmente quebrada (Romanowski et al., 2001; Voet et al., 2002; Rittle et al., 2019). Os metais de transição de forte ligação como ferro, cobre, zinco, manganês, molibdênio e cobalto são frequentemente ligados as metaloproteínas por causa de sua densidade, pequeno raio atômico e interação via eletromagnética e forças eletrostáticas (Saghiri et al., 2015; Vieira et al., 2015).

Desse modo, a metalômica está associada ao proteoma com intuito de verificar a distribuição das espécies metálicas e matalóides, assim como a presença de íons metálicos na composição das biomoléculas em concepções fisiológicas e funcionais, como, por exemplo, as metaloproteínas e as proteínas ligadas a metais (Mounicou et al., 2009; Roberts, 2012; Blindauer, 2019).

Diante deste contexto, o objetivo do presente trabalho foi avaliar o efeito da fonte de Zinco inorgânico fornecida para as abelhas *Apis mellifera* no perfil proteômico e metaloproteômico da geleia real e correlacionar a expressão das metaloproteínas com os diferentes níveis de zinco fornecidos na dieta.

A presente pesquisa resultou no capítulo II, artigo intitulado “Suplementação com fonte inorgânica de zinco no perfil metaloproteômico da geleia real em abelhas *Apis mellifera* L”, publicado na revista “Biological Trace Element Research”, conforme as suas regras de publicação.

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CAPÍTULO 2

“Supplementation with an inorganic zinc source in the metalloproteomic profile of royal jelly in *Apis mellifera* L”

SUPPLEMENTATION WITH AN INORGANIC ZINC SOURCE IN THE METALLOPROTEOMIC PROFILE OF ROYAL JELLY *IN Apis mellifera* L.

Aimê de Almeida Longuini[1] & Gabriel Moreno Martineli[1] & Marcelo Polizel Camilli[1] & Daniel Cavalcante Brambila de Barros[1] & José Cavalcante Souza Vieira[2] & Pedro de Magalhães Padilha[2] & Ricardo de Oliveira Orsi[1]

*** Aimê de Almeida Longuini melonguini@gmail.com**

1 UNESP- São Paulo State University, School of Veterinary Medicine and Animal Science, Botucatu, Brasil

2 UNESP - São Paulo State University, Institute of Biosciences of Botucatu, Botucatu, Brasil

ABSTRACT

This study aimed to evaluate the quality of royal jelly produced by honey bees *Apis mellifera* supplemented with different concentrations of inorganic zinc (Zinc Sulphate monohydrate - 0, 25, 50 and 75 ppm). Was performed two-dimensional electrophoresis for the fractionation of royal jelly proteins, and the zinc level was quantified by the flame atomic absorption spectrometry (FAAS) technique. Proteins were identified by electrospray ionization mass spectrometry (ESI MS MS). Analysis of variance followed by the Tukey test ($P < 0.05$) was used. Supplementation with the mineral zinc positively affected the quantification of proteins for treatments 50 and 75 ppm. However, all treatments independent of zinc concentrations showed fewer protein spots when compared to the control. All zinc-containing proteins were classified as Major Royal Jelly Proteins (MRJPs). The exposure of nursing bees to the mineral zinc in its inorganic form reduced the expression of six different MRJP proteins involved in larval development and expression in the feeding glands of nursing bees (MRJP1, MRJP2, MRJP3, MRJP5 and MRJP7), however promoted an increase in the expression of royal jelly proteins involved in defense systems (MRJP8 and MRJP9). The results demonstrate that vital proteins and metabolic processes are impaired in nursing bees exposed to the mineral zinc in its inorganic form in all doses used affecting nutrition and maintenance of colonies.

Keywords*:* bees, mineral, proteomic, supplementation.

INTRODUCTION

In order for the honey bee colony *Apis mellifera* to express its full productive and reproductive potential, specific nutrients are essential to provide maintenance and survival [1, 2]. Among the main foods consumed by honey bees, royal jelly, formed from the mixture of secretions from the hypopharyngeal glands (clear secretion rich in proteins) and mandibular (milky white component) [3, 4, 5, 6].

Royal jelly is extremely important for the colony, serving as food for worker larvae, queens and drones until the third day of life; after this period, worker larvae and drones receive pollen and nectar / honey as the main food and small amounts of royal jelly; however, the queen larva receives exclusively royal jelly throughout its larval development and adult life, promoting physiological changes such as the

development of structures related to reproduction [4, 7, 8]. For adequate production of royal jelly by young worker bees, they need adequate amounts of protein in their diet [3]. The composition of royal jelly can also vary depending on the age of the larva, environmental conditions, physiological and metabolic state of the nursing bee and pheromones in inducing the production of royal jelly by the hypopharyngeal and mandibular glands [9]. Royal jelly has water-soluble proteins in its composition (Water Soluble Proteins - WSP) that have outstanding nutritional value and are called Major Jelly Proteins or Major Royal Jelly Proteins (MRJPs), where MRJP1, MRJP3 and MRJP5 are responsible for 82% - 90% of total royal jelly proteins [10, 6], whose relative molecular mass is 49 to 87 kDa [11]. So far, nine MRJP proteins have been found in *Apis mellifera* (MRJP1, MRJP2, MRJP3, MRJP4, MRJP5, MRJP6, MRJP7, MRJP8, MRJP9), and an incomplete polypeptide, MRJPψ, encoded by a pseudogene however, little is known about the functions of each [10].

Mineral sources are obtained through pollen, nectar and water, [12] propose a diet with 1000 ppm potassium, 500 ppm calcium, 300 ppm magnesium and 50 ppm sodium, zinc, manganese, iron and copper. [13] suggest 30 ppm of zinc for survival and anti-oxidant activity for honey bees and 60 to 70 ppm for the production of royal jelly. However, there is little knowledge about the need for these micronutrients and their physiological influences on honey bees [14, 15]. Minerals like zinc are fundamental as structural and functional components; in the structural aspect it is present in metalloproteins, and in the functional aspect, it acts as an enzymatic cofactor, participating in metabolism, regulation of gene expression, structural maintenance of biomembranes, immunity and protection against free radicals, protein synthesis, among others [16, 17]. Protein-bound zinc exerts enzymatic catalytic and inhibitory functions, arrays and breakdowns of macromolecular subunit complexes and protein / receptor complex formation [16, 1].

The areas of science called metallome and metalloproteome allow the assimilation between analytical studies and inorganic and biochemical studies [18], therefore the metalloma can present data as (1) metallic or metalloid species divided between the cell compartments of a certain type cell; (2) which biomolecule is incorporated or which bioligand is complexed; (3) the concentrations of the individual metallic species present and (4) the biomolecule structure [18, 19, 20].

Therefore, in order for there to be a greater understanding of the real nutritional needs of the colonies, evaluations using techniques such as proteomics and metalloproteomics will provide an understanding of how minerals linked to protein act in the organism of honey bees. There are still gaps regarding the correct availability of the mineral.

MATERIAL AND METHODS

- Study Site

The experiment was conducted at the apiary in the Beekeeping Production Area of the Lageado Experimental Farm, Faculty of Veterinary Medicine and Animal Science, UNESP, Botucatu, São Paulo, Brazil. It was conducted at the following geographic coordinates: 22° 49′ south and 48° 24′ west. The place is characterized by a humid subtropical climate and an average altitude of 623 m.

- Experimental Groups

Sixteen beehives of Africanized *Apis mellifera* were standardized for the number of breeding (four frames including open and sealed brood) and feeding frames (one), with naturally mated queens and without defined age, and distributed in four treatments (four hives per treatment):

T0Zn: control treatment, without zinc supplementation
T25Zn: supplementation with 25 ppm zinc
T50Zn: supplementation with 50 ppm zinc
T75Zn: supplementation with 75 ppm zinc

The zinc source used was zinc sulphate monohydrate (H_2O_5SZn, 37,4% of zinc) diluted in sugar syrup at a ratio of 1:1 (m/v) commercial crystal sugar and water, supplied using a Boardman feeder (500 mL per week) during July of 2019 (offseason period). The levels provided were confirmed by conducting atomic absorption spectroscopy (FAAS) for zinc level. The following values were obtained: T0Zn, 3.6 ppm; T25Zn, 23.5 ppm; T50Zn, 49.4 ppm; and T75Zn, 75.1 ppm.

- Royal Jelly Collection

Royal jelly was produced according to the method of [21] with modifications, using an excluding screen to separate the queen from artificial cups. Royal jelly was produced in the mini-hive system, composed of two cup frames, containing a batten, with approximately 25 acrylic cups each. To standardize the age of the larvae that were transferred to the artificial domes, two other colonies had their queens trapped in a nest structure, containing the closed sides with an exclusive screen. This process aimed to ensure the queen's posture in the desired frame and the standardization of the larvae age. Larvae aged 3 days were transferred to artificial cups containing a drop of royal jelly diluted in water (1:1 ratio), one larva per cup, using an appropriate brush for transferring. After, the cup bars were introduced in the mini-hive without the queen, and the royal jelly produced was harvested after 72 hours of transfer, stored in vials, and kept in the freezer (below 0 °C) until analysis.

The collection was carried out at the end of the experimental period (four weeks) whit the goal of the close the cycle of the bee (six days of the nurse bees that would feed the larvae; nineteen days of larvae development; and six days of new nurse bees that produced the royal jelly for the experiments, totaling 31 days). For this analysis, the royal jelly, in equal quantities, were mixed and homogenized to obtain a blend for each treatment.

- Protein and metalloprotein analysis of royal jelly

This phase of the project was carried out at the Bioanalytical and Metalloproteomics Laboratory (LBM) of Institute of Biosciences of UNESP of Botucatu, under the supervision and guidance of the Professor Phd. Pedro de Magalhães Padilha and at the Biochemistry Laboratory of the Faculty of Dentistry of the University of São Paulo, Bauru, under the supervision of Professor Phd. Marília Afonso Rabelo Buzalaf.

- Extraction and Precipitation of Royal Jelly Proteins

The proteins from the royal jelly were extracted by placing 250 mg of royal jelly into 2 mL microtubes, and 500 µL of ultrapure water was added, agitated in the vortex, and subjected to centrifugation at 7000 rpm and 4 °C for 30 minutes. The supernatant was collected and subjected to six further rounds of centrifugation at 14000 rpm and 4°C at 5 minutes duration each, to discard any impurity from the sample.

For precipitation, the proteins were added to microtubes containing 100µL of supernatant and 400µL of 80% acetone, rested for 2 hours, and centrifuged again 14000 rpm and 4 °C for 5 minutes. After this process, the supernatant was discarded, and the protein pellets were washed three times with 200 µL of 80% acetone.

The total protein concentration in the royal jelly extracts was determined according to the Biuret method by using bovine serum albumin as a standard [22], briefly described following: An analytical curve with concentrations of 5 to 50 g L^{-1} was constructed from a standard stock solution of bovine albumin 100 g L^{-1}. For samples analysis, protein precipitates were solubilized in 100 µL of 0.50 mol L -1 NaOH. Shortly thereafter, in 5.00 mL glass tubes, 50 µL of protein standard and/or protein extract and 2.50 mL of the Biuret reagent were added. The standard mixture or sample with the reagent was maintained at 32 °C in a water bath for 10 min. After the reaction time, the absorbance readings were measured at 545 nm, using a UV-Visible spectrophotometer.

- Electrophoretic Separations of Protein Fractions

For electrophoresis, the previously obtained protein pellets were solubilised in a solution containing 7 mol L^{-1} urea, 2 mol L^{-1} thiourea, 2% (m/v) 3-[(3-chloroaminopropyl) dimethylammonium]-propan-1 2% (m/v); 0.5% (v/v) ampholytes at pH ranging from 3 to 10; 0.002%bromophenol blue (m/v), and 2.8 mg dithiothreitol (DTT) [22, 23] obtaining a final concentration of de 1.5 mg mL^{-1} of protein. A 250-µL volume of this solution was applied to each Immobiline DryStrip Gel (13 cm) containing immobiline buffer (pH 3–10) and held for 14 hours in box (IPGBOX) at room temperature. Subsequently, isoelectric focusing (IEF) was performed using an EttanIPGphor system (first dimension). The proteins were thus separated according to the isoelectric point following the specifications: step 1: tension – 500V and accumulation of 500Vh; step 2: tension 1000 and accumulation of 800Vh; step 3: tension: 10000 and accumulation of 11300Vh and finally step 4: tension 10000 and accumulation of 3000Vh.

Then the strips were rebalanced in two solutions: reducing, by using 10 mL of solution containing 6 mol L^{-1} urea, 2% SDS (m/v), 30% glycerol (v/v), 50 mmol L^{-1} Tris-HCl (pH 8.8), 0.002% bromophenol blue (m/v), and 2% (m/v) DTT, retaining the proteins in their reduced forms. Alkylating agent, a solution of similar composition, was used, replacing DTT with 2.5% (m/v) iodoacetamide for alkylation of the thiol groups of the proteins and thus preventing their possible re-oxidation. The two steps were performed under stirring for 15 minutes each. These strips were applied to 12.5% (m/v) polyacrylamide gels, and molecular weight standards of 14 to 97 were added alongside each gel; both were sealed with 0.5% w/v agarose solution, and 2D-PAGE was performed in two steps: 30 minutes at 100V and 2 hours at 250V [24].

After the run, the proteins were fixed on the gel by using 10% (v/v) acetic acid solution and 40% (v/v) ethanol for 1 hours, 3 gels were made for Zn analysis and 3 for protein identification. The proteins were stained with colloidal Coomassie blue on the gel for 72 hours. The gels were then washed with ultrapure water, scanned, and analyzed using ImageMaster Platinum software, version 7.0 to obtain the

number of matched protein spots [24]. The images went through an automatic and manual editing process to remove false *spots*, where it is possible to obtain data such as the number of spots per gel, pI, molecular mass of spots, volume of spots and protein mass.

The images treatment was performed for four gels per treatment obtained from the same electrophoretic separation process. Started by adjusting the contrast of the gel image in order to improve visualization by the analyst. Then, a selection of a small region of the gel was made, containing intermediate expression *spots*. The detection parameters used were: smooth (fixes the number of times the program homogenizes the selected region before following detection) – 2; saliency (as it is based on the curvature of the *spot*, filters out real *spots*) – 25; e min area (eliminates smaller regions than indicated) – 5. These parameters were then extended to the entire gel by a program tool for automatic detection.

The correlation obtained between the gels was made by the equivalence between the gel spots (matching), where the *spots* were compared regarding their distribution, volume, relative intensity, isoelectric point (pIs) and molar mass (MM) [24].

- Analysis of Zn by Using FAAS

For the determination of Zn approximately 100 mg of royal jelly were weighed in duplicate and 3 mL of nitric acid and 1 mL of hydrogen peroxide were added; they were digested for 30 minutes in a microwave oven.

The protein spots cut from the gels with the aid of tips were mineralized as follows: 3 protein spots were transferred to 5 mL digestion tubes, each containing 500 µL aliquots of concentrated ultrapure sulfuric acid and 100 µL of 30% (m/m) hydrogen peroxide (triplicate analysis). The set of tubes was placed in a digestion block until complete mineralization of the samples (transparent extract). Next, the acid extracts obtained were added to 5 mL ultrapure water (18.2 MΩ cm^{-1}).

Analytical curves were generated by preparing standard solutions of zinc in 0.10 mol L^{-1} H_2SO_4 medium from the dilution of Titrisol standards containing 1000 mol L^{-1} of analytics. Optimal concentration ranges of the analytical curves were suggested in the equipment manual. For each analytical curve, a blank was prepared. The operating conditions for Zn determinations using FAAS were the same as those described in the manufacturer's manual (Cookbook, Shimadzu AA – 6800, 2000).

- Identification of Proteins by Using ESI MS/MS

The *spots* that presented the zinc were extracted from the gels with the help of a plastic tip, the *spots* were cut from the gels into segments of approximately $1mm^3$, transferred to 0.2 mL tubes containing 1% acetic acid (v/v) and subjected to the following steps: dye removal, protein reduction and alkylation, triptych digestion using trypsin solution 10 ng mL^{-1} and concentrated to a volume of approximately 2 µL. The peptide sequences in the extracts obtained by the triptych digestion process were characterized by ESI MS MS according to the procedure described by [25]. Basically, as aliquots of solutions containing the peptides were analyzed to obtain mass spectra using the system nanoAcquity UPLC-Xevo G2 QTOF-MS (Waters, Machester, UK). Scanning range from 50 to 2000 Da and data were acquired over 20 minutes. Version 3.0 of the ProteinLynx Global Server (PLGS) was used to process and search continuous LC-MSE

data, establishing fixed cysteine carbamidomethylation while the modification and oxidation of methionine, variable, allowing an absent cleavage and a maximum error tolerance of 10 ppm.

Protein identification was obtained with the software's built-in ionic accounting algorithm and searched in the *Apis mellifera* database (UniProtKB/Swiss-Prot em www.uniprot.org). Some parameters commonly used in ESI MS MS were considered, such as the access number of the protein, the identification used in the database (UniProtKB/Swiss-Prot em www.uniprot.org); the score derived from the ion scores and, for a survey that contains a small number of queries, it is the sum of the highest ion score for each distinct sequence; the experimental pI and Mw of the proteins were obtained in the ImageMaster Platinum software for each gel run; the theoretical pI and Mw of the proteins were obtained in the database for each protein found using the protein accession number (UniProtKB/Swiss-Prot emwww.uniprot.org); the coverage used to determine the percentage of residues in each protein sequence that were identified. The FASTA sequences of the detected proteins were obtained and imported into the Blast2GO (B2G) program, which allowed for the separation at three levels (cellular component, molecular function and biological function).

- Statistical Analysis

The results obtained were compared by ANOVA, followed by the Tukey test to verify differences between the means. It was considered as statistically different when $P < 0.05$ [26].

The images of the gels obtained in the fractionation of the proteome of samples of royal jelly were analyzed by the software ImageMaster 2D Platinum 7.0 (GE Healthcare). This software helps to obtain the correlation between the gels (correspondence). This correlation was made by the equivalence between the points of the three repetitions in each repetition of the run, where the points were compared in terms of distribution, volume, relative intensity, isoelectric point and molecular mass by means of GE linear regression.

RESULTS

There was a significant increase in the protein content of royal jelly from colonies that received supplementation with mineral zinc in all concentrations, compared to the control (Table 1).

Table 1. Concentration of total protein (g L^{-1}) obtained in the pellets of the royal jelly extract

Experimental groups	Mean ± SD
T0Zn	13.6 ± 0.1C
T25Zn	18.3 ± 0.7B
T50Zn	21.8 ± 1.4A
T75Zn	20.4 ± 0.6AB

Different letters indicate statistical difference between treatments ($P<0.05$).

The colonies of the treatments supplemented with the mineral zinc presented protein *spots* with important and necessary functions for the life support of the bees, as well as those of the control treatment. However, all colonies of zinc treatments showed fewer protein spots compared to control. 6, 21 and 19 more expressive *spots* were found containing proteins from the MRJP group, for the treatments T25Zn, T50Zn and T75Zn, respectively, compared to the control (Fig. 2, Table 2) the gels presented matches higher than 90%.

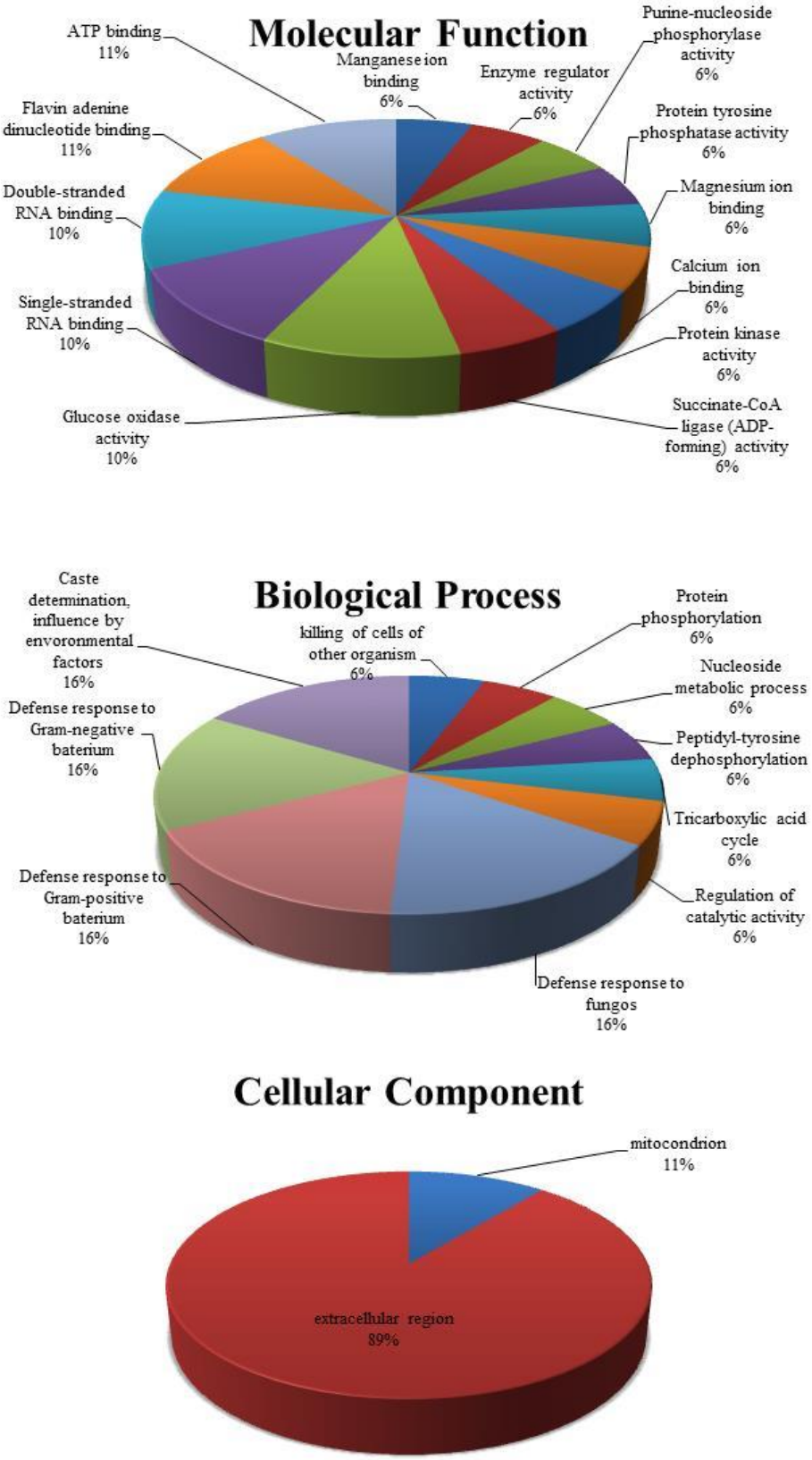

Fig. 1. Analyses of royal jelly proteins (MRJPs) of bees as extracellular components performed by the Blast2Go platform (www.blast2go.com, 2020). The graphs present the molecular functions and biological processes of the proteins found through the proteomics analysis by ESI MS/MS mass spectrometry analysis,

showing the proteins “Major Royal Jelly Protein 1” and “Major Royal Jelly Protein 8” as the main active proteins in royal jelly Up or Down regulated and associated with Zn. For the realization of the graph, the Up FASTA sequences were used.

Table 2 Biological and molecular function and number of proteins identified in spots significantly differentially expressed in the royal jelly of feeding bees fed 25 ppm Zn (T25Zn), 50 ppm Zn (T50Zn), and 75 ppm Zn (T75Zn). Expression analysis of the spots was performed using the ImageMaster Platinum program, with reference to the proteomics of non-mineral zinc bees (T0Zn) with the identification of proteins by ESI-MS/MS. Number of proteins up- and downregulated are presented based on the comparison between bees fed 25 ppm Zn (T25Zn), 50 ppm Zn (T50Zn), and 75 ppm Zn (T75Zn) with without the addition of Zn (control), some spots showed more than one protein.

Difference of expression	**25ppm Zn**		**50ppm Zn**		**75ppm Zn**	
Biological process and Molecular Function (MRJPs)	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**
Interspecies interaction between organisms	5	12	1	6	4	13
Developmental process	5	12	1	6	4	13
Response to stimulus	5	12	1	6	4	13
Metabolic process	-	1	-	-	-	-
Multicellular organismal process	5	12	1	6	4	13
Cell killing	2	11	1	4	-	12
Catalytic activity	-	1	-	-	-	-
Binding	10	19	4	12	10	16
Total	32	80	9	40	26	80

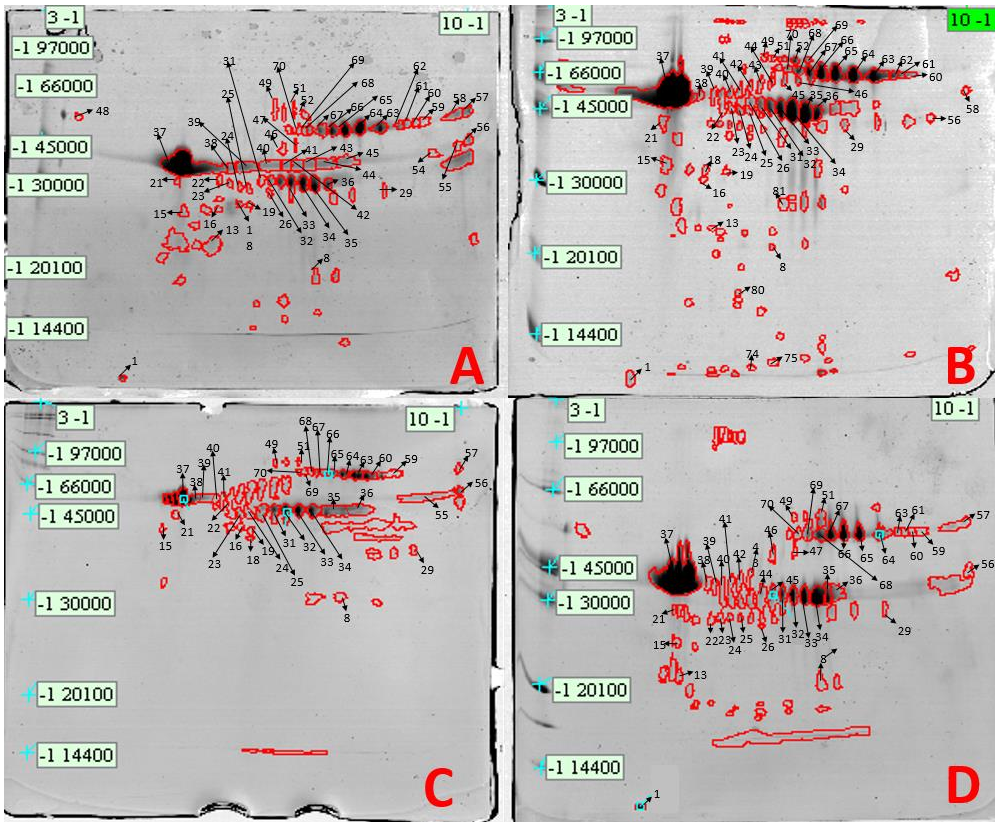

Fig. 2 Representative image of the polyacrylamide gels. The spots that are identified with a number were subjected to ESI MS MS mass spectrometry analysis, and the proteins identified are described in Table 2. Experimental conditions: 12.5% polyacrylamide gel, pH 3 to 10, strips 13 cm. Figure (A) represents bees fed of non-mineral zinc minerals (T0Zn), Figure (B) represents bees fed with addition of 25 ppm Zn (T25Zn), Figure (C) represents bees fed with the addition of 50 ppm Zn (T50Zn) and Figure (D) represents bees fed with addition of 75 ppm Zn (T75Zn).

Table 3 Identification of protein stains with different expression (ANOVA; $P < 0.05$) in the royal jelly of feeding bees fed 25 ppm Zn (T25 Zn), 50 ppm Zn (T50 Zn), and 75 ppm Zn (T75 Zn). Expression analysis of the spots was performed using the ImageMaster Platinum program, with reference to the proteomics of non-mineral zinc bees (T0 Zn) with the identification of proteins by ESI-MS/MS. Positive (+) values show a potential expansion tendency, and negative values (−) show potential reductions in expression. The signal Ø means no expression in zinc-fed groups. These values are evaluated in comparison with the control treatment, without the addition of Zn, some spots showed more than one protein.

Spot ID	Difference of expression 25mg	Difference of expression 50mg	Difference of expression 75 mg	Score	pI/MM theoretical (kDa)	Coverage (%)	Molecular Function
			Major royal jelly protein (D3Y5T0_APIME)				
16	0.0699	-0.0579	Ø	71.7041	6.65 /61.645	5.15	-
18	-0.0552	-0.0752	Ø	465.2162		11.76	
22	-0.0320	0.0593	-0.0412	338.3209		15.07	
23	-0.0630	0.2483	0.0871	998.6307		18.2	
24	0.1088	0.7177	-0.0699	874.3964		18.2	
25	-0.0581	1.0429	0.0960	309.7308		15.07	
31	+0.1773	0.1141	2.6071	166.6245		5.88	
33	+5.0832	4.9902	3.8040	2042.638		2.02	
34	5.5022	3.7882	+6.1014	1308.059		2.02	
38	-0.1873	+5.9685	-0.2290	40.8037		5.88	
39	-0.2173	Ø	-0.1023	729.9177		29.41	
40	-0.6568	-0.4946	-0.2377	673.7283		18.2	
41	-0.1325	0.2105	-0.3297	43.0402		5.33	
48	+0.1281	Ø	0.1730	57.4597		3.31	
56	-0.0374	0.5926	-0.1300	55.4912		7.17	
60	0.2342	+3.9747	0.0382	50.1342		5.33	
65	3.9295	-1.8505	-2.8943	3151.792		34.38	
66	+3.5219	-0.6713	+3.1854	4662.946		41.36	
67	+1.7288	-0.2772	+2.2073	3666.14		40.26	
68	0.5478	0.2768	+2.6341	5309.4		36.21	
69	0.1027	0.1011	+0.9030	1634.099		22.79	
			Major royal jelly protein 1 (MRJP1_APIME)				
1	-0.0232	Ø	1.1935	5518.595	5.10/48.886	49.77	Caste determination influence by environmental factors; defense response to fungus; defense response to Gram-negative bacterium; defense response to Gram-positive bacterium; killing of cells of other organism.
13	-0.0748	Ø	-0.0807	1407.885		35.65	
22	-0.0320	0.0593	-0.0412	203.3688		8.56	
29	-0.1100	0.2836	-0.1062	348.1182		2.55	
37	50.3604	-26.7345	54.2304	1963.112		45.6	
38	-0.1873	+5.9685	-0.2290	12041.74		49.77	
39	-0.2173	Ø	-0.1023	11584.09		56.25	
40	-0.6568	-0.4946	-0.2377	8034.015		51.16	
41	-0.1325	0.2105	-0.3297	5954.192		40.05	
42	0.0862	Ø	-0.1537	4764.696		39.35	
43	-0.1861	Ø	-0.2114	3618.422		33.33	
44	0.0745	Ø	-0.5836	5327.801		37.04	

51	-0.0973	-0.0727	0.0253	1076.779		2.55	
56	-0.0374	0.5926	-0.1300	5212.158		43.06	
65	3.9295	-1.8505	-2.8943	1120.354		19.91	
74	+0.0299	Ø	Ø	143.2723		6.48	
80	+0.0419	Ø	Ø	482.2355		21.76	
			Major royal jelly protein 2 (MRJP2_APIME)				
18	-0.0552	-0.0752	Ø	93.3758	6.83/51.074	2.43	May play an important role in honeybee nutrition. It is found in the royal jelly which is the food of the queen honey bee larva. The royal jelly determines the development of the young larvae and is responsible for the high reproductive ability of the honeybee queen.
22	-0.0320	0.0593	-0.0412	70.2529		2.43	
23	-0.0630	0.2483	0.0871	384.4507		2.43	
24	0.1088	0.7177	-0.0699	163.5405		2.43	
25	-0.0581	1.0429	0.0960	74.9839		2.43	
31	+0.1773	0.1141	2.6071	321.1932		10.4	
33	+5.0832	4.9902	3.8040	11968.04		49.78	
34	5.5022	3.7882	+6.1014	7363.174		50.44	
40	-0.6568	-0.4946	-0.2377	395.2112		18.58	
41	-0.1325	0.2105	-0.3297	21.9523		2.43	
51	-0.0973	-0.0727	0.0253	86.8578		4.65	
65	3.9295	-1.8505	-2.8943	1298.12		2.43	
66	+3.5219	-0.6713	+3.1854	752.6452		2.43	
67	+1.7288	-0.2772	+2.2073	915.2661		2.43	
68	0.5478	0.2768	+2.6341	1186.185		2.43	
69	0.1027	0.1011	+0.9030	435.5278		2.43	
			Major royal jelly protein 3 (MRJP3_APIME)				
16	0.0699	-0.0579	Ø	71.7041	6.47/61.662	5.15	May play an important role in honeybee nutrition. It is found in the royal jelly which is the food of the queen honey bee larva. The royal jelly determines the development of the young larvae and is responsible for the high reproductive ability of the honeybee queen.
18	-0.0552	-0.0752	Ø	465.2162		11.76	
22	-0.0320	0.0593	-0.0412	338.3209		15.07	
23	-0.0630	0.2483	0.0871	998.6307		18.2	
24	0.1088	0.7177	-0.0699	881.9487		21.14	
25	-0.0581	1.0429	0.0960	309.7308		15.07	
31	+0.1773	0.1141	2.6071	166.6245		5.88	
33	+5.0832	4.9902	3.8040	2042.638		2.02	
34	5.5022	3.7882	+6.1014	1308.059		2.02	
38	-0.1873	+5.9685	-0.2290	40.8037		5.88	
39	-0.2173	Ø	-0.1023	729.9177		29.41	
40	-0.6568	-0.4946	-0.2377	673.7283		18.2	
41	-0.1325	0.2105	-0.3297	43.0402		5.33	
48	+0.1281	Ø	0.1730	57.4597		3.31	
56	-0.0374	0.5926	-0.1300	55.4912		7.17	
60	0.2342	+3.9747	0.0382	50.1342		5.33	
65	3.9295	-1.8505	-2.8943	3161.514		37.13	

66	+3.5219	-0.6713	+3.1854	4662.946		41.36	
67	+1.7288	-0.2772	+2.2073	3666.14		40.26	
68	0.5478	0.2768	+2.6341	5309.4		36.21	
69	0.1027	0.1011	+0.9030	1634.099		22.79	
			Major royal jelly protein 5 (MRJP5_APIME)				
13	-0.0748	Ø	-0.0807	327.1173	5.95/70.236	6.52	May play an important role in honeybee nutrition. It is found in the royal jelly which is the food of the queen honey bee larva. The royal jelly determines the development of the young larvae and is responsible for the high reproductive ability of the honeybee queen.
22	-0.0320	0.0593	-0.0412	48.6069		1.84	
29	-0.1100	0.2836	-0.1062	348.1182		5.85	
33	+5.0832	4.9902	3.8040	44.4162		1.67	
34	5.5022	3.7882	+6.1014	314.067	6.12/70.182	3.68	
38	-0.1873	+5.9685	-0.2290	3529.314	5.95/70.236	1.84	
39	-0.2173	Ø	-0.1023	2645.292		1.84	
40	-0.6568	-0.4946	-0.2377	1225.509		3.18	
41	-0.1325	0.2105	-0.3297	1539.617		1.84	
42	0.0862	Ø	-0.1537	483.2089		1.84	
43	-0.1861	Ø	-0.2114	910.464		1.84	
44	0.0745	Ø	-0.5836	1218.444		1.84	
49	-0.0248	0.0895	0.0358	102.1785	6.12/70.182	8.03	
51	-0.0973	-0.0727	0.0253	1661.632	5.95/70.236	20.74	
56	-0.0374	0.5926	-0.1300	1108.521		1.84	
65	3.9295	-1.8505	-2.8943	476.1633		1.84	
			Major royal jelly protein 7 (Q6IMJ9_APIME)				
33	+5.0832	4.9902	3.8040	44.4162	4.90/50.541	2.26	-
51	-0.0973	-0.0727	0.0253	27.8727		2.26	
			Major royal jelly protein 8 (Q6TGR0_APIME)				
23	-0.0630	0.2483	0.0871	77.8079	6.00/46.956	1.44	-
65	3.9295	-1.8505	-2.8943	139.8646		1.44	
67	+1.7288	-0.2772	+2.2073	157.1417		1.44	
68	0.5478	0.2768	+2.6341	92.6368		1.44	
69	0.1027	0.1011	+0.9030	17.6203		1.44	
			Major royal jelly protein 9 (B3GM12_APIME)				
31	+0.1773	0.1141	2.6071	22.6794	8.70/48.688	1.65	-
33	+5.0832	4.9902	3.8040	279.0468		1.66	
34	5.5022	3.7882	+6.1014	308.985		1.66	

DISCUSSION

In off-season periods, the reduction of available food resources can impair the balance of nutrients necessary for the maintenance of colonies and the proper functioning of bee metabolism [1, 2]; thus, the supply of artificial diets by beekeepers is an important strategy for maintaining colonies [27, 28, 29, 30,

31], carried out with energy or protein foods. However, little is known about the effects of micronutrients on the bee's diet, such as the mineral zinc.

The need for honey bees for the mineral zinc is met by harvesting nectar and pollen, where its concentration may vary according to the bee pasture available around the apiary (0.012 to 173.77 mg kg^{-1} in honey and from 5.1 to 340.0 mg kg^{-1} in bee pollen) [13, 32]. In this work, zinc concentrations were used based on studies such as [12] that propose a diet with 50 ppm of zinc and [13], which suggest 30 ppm zinc for honey bee survival and anti-oxidant activity and 60 to 70 ppm for the production of royal jelly, suggesting that the supply of 60 mg kg^{-1} of zinc in the pollen harvested by the honey bees would be sufficient to meet the nutritional requirements of this mineral.

It was observed that supplementation of *Apis mellifera* bee colonies with the mineral zinc in its inorganic source promoted an increase in the protein content of royal jelly in all concentrations used. Even though zinc supplementation did not influence the binding of this mineral to royal jelly proteins, evidenced by the reduction in the number of protein spots, possibly due to the lower bioavailability of inorganic zinc [33] there is an increase the expression of proteins involved in defense systems, possibly due to the effects of Zn supplementation.

The Major Royal Jelly Proteins represent about 80% of the soluble proteins of royal jelly and it is estimated that these proteins are responsible for the physiological actions of the queen due to the large amount of amino acids [34, 5, 35, 36, 37]. All zinc-linked protein spots belonged to the group of proteins called Royal Jelly Main Proteins (MRJPs), where major Major royal jelly protein (D3Y5T0_APIME), royal jelly protein 1 (MRJP1_APIME), major royal jelly protein 2 (MRJP2_APIME), major royal jelly protein 3 (MRJP3_ APIME), major royal jelly protein 5 (MRJP5_APIME), major Major royal jelly protein 7 (Q6IMJ9_APIME), royal jelly protein 8 (Q6TGR0_APIME) and major royal jelly protein 9 (B3GM12_APIME) were expressed more or less than according to the supplied zinc level, as can be seen in Fig. 1, Table 2.

These proteins play important roles in honey bees, as defense against gram-positive and gram-negative bacteria, defense against fungi and biological balance (Fig. 1). However, only the T25 Zn treatment for the MRJP9 protein and the T75 Zn treatment for the MRJP8 and MRJP9 proteins showed greater expression compared to the T0Zn treatment. Most of the proteins found in royal jelly had a positive correlation in the treatments T25 Zn, T50 Zn and T75 Zn, and these proteins were less expressed compared to those in the T0Zn treatment for the other proteins found (Table 3).

The proteins belonging to the MRJPs group contain the ten essential amino acids needed by honey bees, they are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, where MRJP8 contains 49.5% and MRJP9 contains 47.3 % of amino acid content [34, 4, 35, 37]. The MRJP8 and MRJP9 proteins considered ancestors of the MRJPs family do not have a nutritional role, they are found more frequently in the thoracic limbs and abdomen, being part of the components of honey bee venom and immunosensitizing agent [38, 6, 35].

Despite having low expression when compared to the T0Zn group, the MRJP3 protein group showed a higher amount of zinc-linked spots for the T25Zn group when compared to the other proteins and treatments. This protein may be responsible for transmitting immunity from nurse bees to the larvae for the purpose of fighting pathogens [34, 6, 35].

There is little information regarding the correct availability of the mineral zinc in the nutrition of bees and its appropriate level to be applied. However, the composition of royal jelly can be affected according to the available food source. Therefore, metalloproteomics is an important tool in understanding royal jelly, highlighting the proteins associated with the mineral and showing its importance to honey bees.

CONCLUSIONS

It was concluded that the exposure of nursing bee to the inorganic zinc mineral form reduced the expression of six of the Major Royal Jelly Proteins (Down regulation: MRJP, MRJP1, MRJP2, MRJP3, MRJP5 and MRJP7), however it promoted an increase in the expression of jelly proteins involved in defense systems (Up regulation: MRJP8 e MRJP9). Our results demonstrate that vital proteins and metabolic processes are impaired in nursing bees exposed to the mineral zinc in its inorganic form in all doses used affecting nutrition and maintenance of colonies.

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AUTHORS CONTRIBUITION

All authors have contributed equally to the work.

CONFLICT OF INTEREST

The authors declare that they have no potential conflict of interest in relation to the study in this paper.

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CAPÍTULO 3

IMPLICAÇÕES

Nota-se neste estudo que durante o período de entressafra a exposição de abelhas nutrizes ao mineral zinco em sua forma inorgânica reduziu a expressão de seis das principais proteínas da geleia real, interferindo de forma negativa, demonstrando que proteínas vitais e processos metabólicos são prejudicados em abelhas nutrizes expostas ao mineral zinco em sua forma inorgânica em todas as doses utilizadas afetando a nutrição e manutenção de colônias, entretanto promoveu aumento na expressão de proteínas da geleia real envolvidas em sistemas de defesa.

A carência de estudos na área de nutrição apícola associada ao mineral zinco foi um impasse para a determinação das doses de zinco a serem fornecidas a campo, assim como a carência na parte proteomica associada a proteínas que pouco sabemos sobre suas funções, portanto, existe um hiato grande em questão de nutrição apícola associada a minerais como o zinco necessitando de pesquisas afim de determinar níveis seguros de inclusão de minerais na dieta das abelhas.

Espera-se que esse trabalho possa colaborar com elucidações e bases na nutrição das abelhas relacionadas aos níveis de zinco suplementados, afim de contribuir com futuros estudos na estimação da real exigência do mineral e seus efeitos durante o período de entressafra em abelhas *Apis mellifera*.