UNIVERSIDADE ESTADUAL PAULISTA “Júlio de Mesquita Filho”

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

CÂMPUS DE BOTUCATU

EFEITO DA SUPLEMENTAÇÃO DE ZINCO NO PERFIL PROTEÔMICO E METALÔMICO DA GELEIA REAL E NO TRANSCRIPTOMA DE ABELHAS *Apis mellifera* L.

MARCELO POLIZEL CAMILLI

BOTUCATU – SP

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MARCELO POLIZEL CAMILLI

ORIENTADOR: Prof. Dr. Ricardo de Oliveira Orsi

Tese apresentada ao Programa de PósGraduação em Zootecnia como parte das exigências obtenção do título de Doutor em Zootecnia

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"Nem tudo que reluz é ouro,
Nem todos os que vagueiam estão perdidos"

J. R. R. Tolkien

DEDICATÓRIA

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BIBLIOGRAFIA DO AUTOR

Marcelo Polizel Camilli nasceu na cidade de Bocaina – SP no dia 12 de setembro de 1991. Mudou-se para Dois Córregos aos sete meses de idade com sua mãe Marta Rita Polizel Camilli e seu irmão Marco Antonio Polizel Camilli.

Aos doze anos de idade começou a acompanhar seu avô Antonio Luis Camilli em seu apiário, despertando seu interesse no mundo das abelhas. Durante a adolescência sempre teve contato com a natureza, acampando e pescando com seus amigos, o que fez com que acendesse uma verdadeira paixão pelos seres vivos e escolhesse sua futura carreira profissional.

Em 2011, mudou-se para Botucatu – SP após ingressar no curso de Ciências Biológicas do Instituto de Biociências (IB) da Universidade Estadual Paulista (UNESP). No ano de 2014 teve contato com a sala de aula, tornando-se professor eventual da Escola Estadual Profa. Sophia Gabriel de Oliveira, por dois anos.

Em Julho de 2016 se interessou pelas linhas de pesquisa do Núcleo de Ensino, Ciência e Tecnologia em Apicultura Racional (NECTAR) da UNESP Botucatu e realizou um estágio no setor de apicultura. Graduou-se em Ciências Biológicas pelo IB, UNESP Botucatu em Dezembro de 2016 e ingressou no Programa de Pós-Graduação em Zootecnia da FMVZ, UNESP – Botucatu sob orientação do Prof. Dr. Ricardo de Oliveira Orsi. Seu trabalho de mestrado teve como objetivo avaliar os efeitos da suplementação proteica no desenvolvimento das glândulas mandibulares de abelhas *Apis mellifera*.

Em janeiro de 2019 ingressou no doutorado sob orientação do Prof. Dr. Ricardo de Oliveira Orsi, continuando seus estudos na área de nutrição de abelhas *Apis mellifera*.

RESUMO GERAL

As abelhas *A. mellifera* possuem necessidades específicas de nutrientes para que possam desenvolver todo o seu potencial produtivo e reprodutivo, visando a sobrevivência e manutenção da colônia. Neste projeto avaliamos duas fontes de zinco (orgânico e inorgânico), em diferentes concentrações e seus reflexos no perfil metaloproteômico da geleia real e no transcriptoma de abelhas com 6 dias de idade. Foram utilizadas 35 colônias de abelhas *A. mellifera*, distribuídas aleatoriamente nos seguintes tratamentos (05 colmeias por tratamento): controle: xarope de açúcar sem suplementação de zinco; ZNI25: xarope de açúcar suplementado com 25 ppm de Zn inorgânico; ZNI50: xarope de açúcar suplementado com 50 ppm de Zn inorgânico; ZNI75: xarope de açúcar suplementado com 75 ppm de Zn inorgânico; ZNO25: xarope de açúcar suplementado com 25 ppm de Zn orgânico; ZNO50: xarope de açúcar suplementado com 50 ppm de Zn orgânico; ZNO75: xarope de açúcar suplementado com 75 ppm de Zn orgânico. A fonte de zinco inorgânico foi o sulfato de zinco monohidratado (37,4% de zinco) e a fonte orgânica de zinco metionina (16% de zinco), diluída em xarope de açúcar. Os níveis reais de zinco foram determinados por espectrometria de absorção atômica (FAAS). As abelhas A. mellifera recém-emergidas foram marcadas ao final de 30 dias de suplementação com caneta atóxica, na região pronoto e suas coletas para análise do transcriptoma foram realizadas aos 6 dias de idade em sua fase de nutriz, com exatamente 36 dias de experimento. Para a análise do metaloproteoma da geleia real, foi induzido a produção de geleia real pelo método de transferência de larvas. Assim que coletada, o teor proteico das amostras era mensurado por espectrômetro de UV visível, as proteínas eram separadas pela eletroforese bidimensional (2D page) e as metaloproteínas separadas por espectrômetro de absorção atômica. Todas as funções biológicas associadas as metaloproteínas que continham zinco foram identificados por espectrômetro de absorção atômica. Nossos resultados sugerem que as menores concentrações de zinco orgânico (ZNO 25) alteram o maior número de expressão diferencial de proteínas da geleia real e também aumentam a concentração proteica das amostras. Em relação as análises do transcriptoma, nossos resultados sugerem que os maiores níveis de zinco inorgânico (ZNI 75) alteram a maior via de genes relacionados a importantes processos fisiológicos, entretanto, somente as menores concentrações de zinco de fonte orgânica (ZNO25) apresentaram grande aumento de genes diferencialmente expressos quando relacionados ao grupo controle (ZN0).

Palavras-chave: Abelhas / minerais / nutrição/ proteômica/ transcriptoma /

ABSTRACT

A. mellifera bees have specific nutrient needs so that they can develop their full productive and reproductive potential, aiming at colony survival and maintenance. In this project, we evaluated two sources of zinc (organic and inorganic), at different concentrations and their effects on the metalloproteomic profile of royal jelly and the transcriptome of 6-day-old bees. Thirty-five colonies of A. mellifera bees were used, randomly distributed in the following treatments (05 hives per treatment): control: sugar syrup without zinc supplementation; ZNI25: sugar syrup supplemented with 25 ppm inorganic Zn; ZNI50: sugar syrup supplemented with 50 ppm inorganic Zn; ZNI75: sugar syrup supplemented with 75 ppm inorganic Zn; ZNO25: sugar syrup supplemented with 25 ppm organic Zn; ZNO50: sugar syrup supplemented with 50 ppm organic Zn; ZNO75: Sugar syrup supplemented with 75 ppm organic Zn. The inorganic zinc source was zinc sulfate monohydrate (37.4% zinc) and the organic zinc source methionine (16% zinc), diluted in sugar syrup. Actual zinc levels were determined by atomic absorption spectrometry (FAAS).

The newly emerged A. mellifera bees were tagged at the end of 30 days of supplementation with a non-toxic pen, in the pronotum region, and their collections for transcriptome analysis were carried out at 6 days of age in their nursing phase, with exactly 36 days of the experiment. . For the analysis of the royal jelly metalloproteome, royal jelly production was induced by the larval transfer method. Once collected, the protein content of the samples was measured by a visible UV spectrometer, the proteins were separated by two-dimensional electrophoresis (2D page) and the metalloproteins were separated by an atomic absorption spectrometer. All biological functions associated with zinc-containing metalloproteins were identified by an atomic absorption spectrometer. Our results suggest that the lower concentrations of organic zinc (ZNO 25) alter the greater number of differential expressions of royal jelly proteins and also increase the protein concentration of the samples. Regarding the transcriptome analyses, our results suggest that the highest levels of inorganic zinc (ZNI 75) alter the largest pathway of genes related to important physiological processes, however, only the lowest concentrations of zinc from an organic source (ZNO25) showed a large increase of differentially expressed genes when related to the control group (ZN0)

Keywords: Bees / minerals / nutrition / proteomics / transcriptome /

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CAPÍTULO 1

CONSIDERAÇÕES INICIAIS

Abelhas *Apis mellifera*

1.1 Histórico da apicultura no mundo

As abelhas *A. mellifera* surgiram há aproximadamente 100 milhões de anos, no período Cretáceo, em que as plantas angiospermas e algumas espécies de vespas passavam por um processo de co-evolução e, por pressões ambientais, foram selecionados os indivíduos que se alimentavam exclusivamente de néctar e pólen, dando origem as primeiras espécies de abelhas (DANFORTH, 2007; GUPTA, 2014).

Os seres humanos começaram a se relacionar com as abelhas a aproximadamente 15.000 anos a.C., no período paleolítico. Pinturas rupestres ilustram a rusticidade da prática da extração de mel (figura1). Após muitos anos da extração de mel predatória o ser humano começou a criar abelhas em colmeias fabricadas com palha, argila e madeira, agrupando-as de maneira a facilitar o manejo, surgindo os primeiros apiários. Os registros dessas criações datam de 1450 a.C. na região do Egito Antigo (figura 2) (CRANE, 1999).

Figura 1. Pintura rupestre ilustrando relação do homem com as abelhas. http://tpa.sapo.ao/noticias/ciencia/o-que-faz-o-mel-ser-eterno-e-nao-estragar.

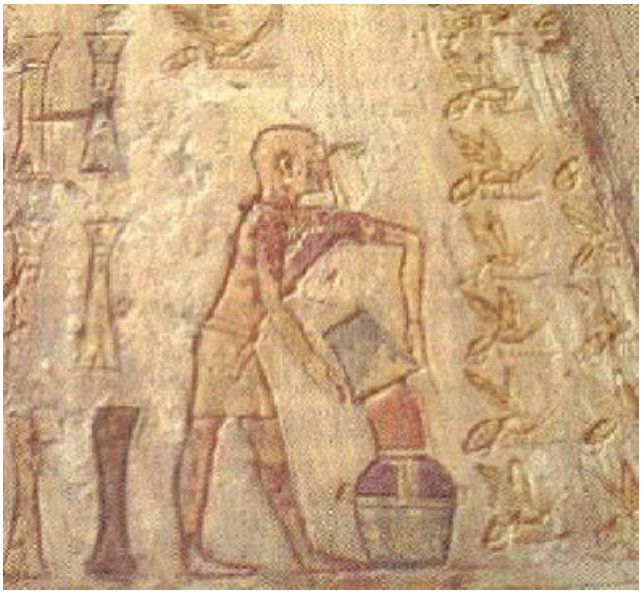

Figura 2. Apicultura no Egito antigo.
https://bdtd.ibict.br/vufind/Record/UFCG_ee186c4da3fe705e9e0c88b30ad4fd57

Com o passar dos anos, a apicultura se desenvolveu de maneira racional, buscando o bem-estar animal, produtividade e longevidade da colônia. O marco da apicultura racional foi em 1851, quando o norte-americano Lorenzo Lorain Langstroth que padronizou a colmeia de madeira com dimensões apropriadas e quadros móveis, surgindo o "espaço abelha", uma lacuna com medidas de 4,7 a 9,6 mm entre os quadros, facilitando a locomoção das abelhas e organizando a construção de favos, o que se tornou o padrão da apicultura universal (CRANE, 1999).

A apicultura racional permite o manejo racional com a colmeia de abelhas *A. mellifera*, permitindo a exploração de recursos como o pólen, mel, própolis, apitoxina e geleia real sem que abale a estrutura e harmonia da colônia (CUNHA, 2013).

No Brasil, a apicultura se iniciou em 1839 quando o imperador Dom Pedro II autorizou o Padre Antonio Carneiro a trazer de Portugal as colônias de abelhas europeias *A. mellifera mellifera.* O objetivo inicial era fornecer mel para a corte real e produzir cera para a fabricação de velas. Outras subespécies foram introduzidas no Brasil durante o final do século XIX: as *A. mellifera ligustica*, *A. mellifera carnica* e *A. mellifera caucasica* disseminando a espécie por todo o território nacional (WIESE, 2005).

Em 1956, visando o melhoramento genético das abelhas melíferas no Brasil, o professor Warwick Kerr introduziu no país rainhas africanas da subespécie A. mellifera

scutellata. As colônias africanas foram acidentalmente expostas ao meio e cruzaram naturalmente com as colônias de abelhas europeias, gerando um polihíbrido denominado abelha africanizada. Como características, essas abelhas apresentam comportamento altamente defensivo, o que levou à uma adaptação da forma com que os apicultores manejavam suas colônias, principalmente no uso de equipamento de proteção individual mais robusto e maior necessidade no uso da fumaça (WINSTON, 1992; DE JONG, 1996). Além disso, as abelhas africanizadas possuem alta capacidade de migração (enxameação), o que causou sua rápida dispersão por grande parte do continente americano, chegando até aos EUA em 1994 (MELLO, et al. 2003).

1.3 Características gerais das abelhas *Apis mellifera*

As abelhas *Apis mellifera*, 1758 são insetos representantes da ordem Hymenoptera, da família Apidae (WINSTON, 2003; GUPTA 2014), eussociais, representando uma sociedade complexa com uma comunicação extremamente sofisticada.

As colônias de abelhas *A. mellifera* possuem milhares de indivíduos com dimorfismo sexual, funções bem definidas e se dividem em três castas: rainha, zangões e operárias (figura 3). rainha é a única fêmea fértil da colônia, responsável pela postura de ovos que garantem a renovação da população da colônia e pela produção de feromônios, substâncias fundamentais para manutenção da organização social e homeostase da colônia (SEELEY, 1995). A cópula ocorre durante o voo nupcial onde a rainha se acasala em média com 12 zangões, preenchendo sua espermateca com espermatozoides para manter a taxa de postura de óvulos fertilizados durante toda sua vida (ESTOUP, SOLIGNAC & CORNUET, 1994).

A)  **B)**  **C)** 

Figura 3. Castas encontradas em uma colônia de *Apis mellifera*. **A)** rainha **B)** operária **C)** zangão. Adaptado de: https://www.insetologia.com.br/2017/02/.

O número de zangões na colmeia varia de dependendo de fatores climáticos e recursos disponíveis, podendo chegar a 400 indivíduos e vivem até 80 dias. Os zangões nascem de óvulos não fertilizados e não apresentam outra função a não ser de fecundação da rainha. Após a cópula perdem seu órgão reprodutor e morrem por perda de hemolinfa (PAGE e PENG 2001).

A casta mais numerosa em uma colônia é de abelhas operárias; vivem cerca de seis semanas e são responsáveis pela maior parte das tarefas realizadas na colônia, de acordo com sua idade fisiológica ou necessidade da colônia. Nos primeiros três dias de vida as abelhas operárias realizam a limpeza interna da colmeia. Do 4º ao 12º são denominadas nutrizes, produzem geleia real e alimentam as larvas e rainha, do 13º ao 18º dia produzem cera, do 19º ao 20º protegem a colmeia de invasores e a partir do 21º dia as abelhas operárias vão ao campo coletar recursos como pólen, néctar, resina e água (SEELEY, 1995; AMDAM; PAGE, 2010).

1.4 Importância global

A produção mundial de mel gira em torno de 1,67 milhões de toneladas segundo FAO (2017), sendo a produção brasileira em média 46 mil toneladas (IBGE, 2019). Além da produção de mel a apicultura também possibilita a exploração de própolis, pólen, cera, apitoxina e geleia real, que apresentam importância farmacêutica, nutricional e gera empregos para milhares de cidadãos (CUNHA, 2013).

As abelhas melíferas também se destacam pela eficiência, ampla distribuição, possibilidade de transporte das colônias para efetuar serviços de polinização (PAUDEL,

2015). O uso de colônias de *A. mellifera* aumenta em 80% a produção de sementes em espécies como a maçã e o melão (WITTER e BLOCHTEIN, 2003), melhorando qualidade do fruto (KLATT et al., 2014). Mundialmente, em torno de 1500 culturas são dependentes de insetos para a polinização (KLEIN et al., 2007), sendo o valor da polinização realizada pelas abelhas para a agricultura mundial é estimado em 200 bilhões de dólares por ano (LEBUHN et al., 2013).

Além disso, as abelhas contribuem diretamente para a manutenção de ecossistemas, nas áreas de vegetação nativas, a polinização que garante o sucesso reprodutivo de 63% das espécies vegetais é realizada por estes insetos (OLLERTON et al., 2011). Atualmente, a diminuição na população de polinizadores tem prejudicado a polinização de diversas culturas agrícolas e comprometendo a manutenção e regeneração de áreas de matas nativas. Neste sentido, o manejo adequado de colônias de abelhas *A. mellifera* é indispensável para suprir a necessidade de polinizadores nas mais diversas culturas agrícolas, garantindo a produção de alimentos e a manutenção da biodiversidade de todo o planeta. (GARIBALDI et al., 2014; GOULSON et al., 2015).

1.5 Geleia real

A geleia real é um alimento rico utilizado na nutrição de larvas e rainha (MICHENER, 1974; CRAILSHEIM, 1992; PINTO et al., 2012). Essa substância é composta a partir de uma mistura de secreções das glândulas hipofaringeanas e mandibulares, localizadas na cabeça das operárias de *A. mellifera* (Coelho et al., 2008). Estas secreções glandulares são produzidas pelas abelhas nutrizes entre o 4° ao 12° dia de vida, sendo que, a glândula mandibular secreta um componente branco leitoso e a hipofaringeana produz uma secreção de coloração clara e rica em proteínas (Zheng et al., 2010).

Segundo Qu et al., 2008, a composição da geleia real varia com a idade e subespécie das abelhas nutrizes, bem como o tipo de alimento consumido e a condição sazonal, sendo então encontrados vários compostos como água (50-70%), proteínas (9- 18%), carboidratos (7-18%), lipídios (3-8%, principalmente o 10-hidroxi-2-decenoico), sais minerais (0,8-3%, sendo encontrados em ordem decrescente : K, Ca, Na, Mg, Zn, Fe, Cu e Mn) e vitaminas (principalmente tiamina, niacina, e riboflavina) (Ramadan e Al-Ghamdi, 2012)

Dentre os compostos da geleia real as proteínas se destacam, podendo chegar de 9 a 15% do seu peso total. Diversos estudos têm identificado e caracterizado essas proteínas, das quais se destacam a família das MRJPs (major royal jelly proteins), representando um total de 82% de todas as proteínas hidrossolúveis e cerca de 90% do total de proteínas da geleia real (SCHMITZOVA et al., 1998).

Até o presente momento, foram identificadas nove proteínas das MRJPs em *A. mellifera* (MRJP1, MRJP2, MRJP3, MRJP4, MRJP5, MRJP6, MRJP7, MRJP8, MRJP9), (DRAPEAU et al., 2006). A MRJP1 é a glicoproteína dominante da geleia real, representando 48% do total de proteínas solúveis (SIMUTH et al., 2004), e juntamente com a MRJP2, MRJP3 e MRJP5 são consideradas as reservas biológicas de nitrogênio e aminoácido para o rápido desenvolvimento das abelhas. As MRJP6, 7, 8 e 9, não possuem funções nutricionais bem definidas, uma vez que não são exclusivamente encontradas neste alimento secretado (MAJTAN et al., 2006).

Diferente do mel e pólen, a geleia real é um alimento que não se encontra estocado nas colônias, para sua obtenção, é necessário o uso de um método para que sua produção seja induzida. Doolittle (1899) foi o pioneiro em publicar seu método de produção de geleia real, que consiste na transferência de larvas jovens (menos de 24 horas de idade) dos favos das colônias para cúpulas artificiais contendo uma pequena gota de geleia real diluída em água. Essas cúpulas são fixadas em quadros e introduzidas na parte central dos enxames, induzindo a colônia a criarem uma nova rainha, estocando geleia real nas próprias cúpulas. 72 horas após a transferência as cúpulas são coletadas, as larvas descartadas e a geleia real colhida.

2 Alimentação das abelhas *A. mellifera*

2.1 Exigência nutricional

As abelhas *A. mellifera* possuem uma exigência de nutrientes comum a todas as espécies animais: carboidratos, proteína, lipídeos, vitaminas, minerais e água. A fonte de alimentos das abelhas melíferas se resume em néctar e pólen. O néctar é a fonte de energia (carboidratos) e o pólen fonte de proteínas, minerais, vitaminas e lipídeos (COUTO, 1996).

O pólen é constituído de 10 a 36% de conteúdo proteico, 20 a 40% de carboidratos, 30% de água, 1 a 20% de lipídios e 1 a 7% de minerais, além de resinas, vitaminas, enzimas e coenzimas (MORETI, 2006), variando de acordo com sua origem

botânica. O consumo de pólen está intimamente relacionado ao desenvolvimento de glândulas e acúmulo de gorduras corporais (CAMPANA e MOELLER, 1977; MARCHINI et al., 2006).

Além do conteúdo proteico, o pólen é reconhecido por ser fonte de vitaminas e antioxidantes, como β-caroteno, pró-vitamina A, vitaminas C e E, vitaminas do complexo B (Riboflavina (Vitamina B2), e Niacina (Vitamina B3), fundamentais no processo metabólico de proteínas, carboidratos, lipídeos, carboidratos. Nutrientes esses necessários para respiração celular, desenvolvimento corporal, colaboram com o funcionamento do sistema nervoso, e na produção de ácido clorídrico para o sistema digestivo (MELO et al., 2009).

Apesar dos alimentos energéticos e proteicos apresentarem enfoque na alimentação das abelhas, nutrientes como vitaminas e minerais são indispensáveis para o desenvolvimento das colônias (HAYDAK, 1970; EYER, 2016). Os íons metálicos constituam são essenciais, atuando como componentes estruturais e em diversos processos vitais (SILVA et al., 2013). Na parte estrutural, destacam seu papel como integrantes de compostos orgânicos corporais e na parte funcional, como catalisador de sistemas enzimáticos, por exemplo.

2.2 Zinco

O zinco é um metal de transição e foi reconhecido pela primeira vez como essencial para sistemas biológicos em 1869. O zinco é um nutriente essencial para o metabolismo animal, pois participa de importantes funções no organismo (KLASING 1998). Este mineral pode ser considerado o mais importante mineral metabolicamente ativo e sua deficiência prejudica a maioria das rotas metabólicas, uma vez que tem participação em diversos processos enzimáticos (KLASING 1998).

O zinco é essencial para o funcionamento celular, atuando 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 (FAA et al., 2008). Por volta de 40% das proteínas e enzimas necessitam da presença de um íon metálico para que possam realizar sua atividade biológica (ROMANOWSKI et al., 2001; GARCIA et al., 2006).

Dentro das células, o zinco é o mineral que está presente em maior quantidade, exercendo papel fundamental em mais de 300 classes de enzimas. De todos os minerais que se ligam a proteínas, o zinco é o mais abundante (SHI e CHANCE, 2011). Por isso

o zinco tornou-se um foco de investigação nos processos de controle da expressão gênica e outros mecanismos biológicos (VALLE et al, 1993).

Ligado às proteínas, o zinco participa no estabelecimento permanente e modulação transitória das interações proteína-proteína, exercendo funções catalíticas e inibitórias enzimáticas, arranjos e desarranjos de complexos de subunidades macromoleculares e formação de complexo proteína/receptor (MARET, 2004). Diante disso, alterações da concentração de zinco disponíveis na dieta podem alterar diversas vias metabólicas, de desenvolvimento e na fisiologia dos mais distintos seres vivos.

3.0 Proteômica

A proteômica é a área da ciência que estuda o proteoma, definido como a composição de todas as proteínas expressas a partir do genoma de um organismo (WESTERMEIER & NAVEN, 2002). Os objetivos da proteômica são quantificar e identificar todas as proteínas presentes em determinada amostra, abordando sua expressão e alterações em relação a um intervalo de tempo, o que torna a seu estudo mais desafiador em relação ao sequenciamento de um genoma, que é estático (ZHANG et al., 2015).

Todo organismo está exposto a alterações no ambiente, condições patológicas, presença de substâncias tóxicas, ausência de determinados nutrientes na alimentação, processos de desenvolvimento, entre outras condições afetam diretamente no perfil do proteoma, atribuindo alta complexidade em seu estudo (ALBERTS et al., 2002). O número de proteínas existentes numa amostra, sua estrutura tridimensional, interações entre moléculas, que quando alteradas modificam sua função biológica (ALBERTS et al., 2002).

Atualmente, existem duas principais técnicas para a análise do proteoma: a quantitativa, chamada de eletroforese bidimensional (2D-PAGE; 2-Dimensional Polyacrylamide Gel Electrophoresis) e a qualitativa, com o nome de espectrometria de massas (MORAES et al., 2013). Na técnica 2D-PAGE, as proteínas são submetidas a dois processos consecutivos de separação, baseados em propriedades diferentes das proteínas. Durante a primeira dimensão, denominada focalização isoelétrica (IEF), as proteínas são separadas em um gel de poliacrilamida, formando um gradiente de pH, e migram até atingirem uma posição estacionária onde possuam carga líquida zero (ponto isoelétrico). Na segunda dimensão, as proteínas separadas pela IEF são submetidas a uma eletroforese

desnaturante em gel de poliacrilamida, que separa as proteínas de acordo com suas massas moleculares (MORAES et al., 2013).

A técnica de espectrometria de massas é uma técnica capaz de quantificar e determinar a sequência de aminoácidos dos peptídeos, identificando as proteínas que constituem a amostra. O espectrômetro de massas é a ferramenta utilizada para analisar íons em fase gasosa, este equipamento consiste em três módulos principais: uma fonte da ionização que converta moléculas da fase de gás em íons, um analisador em massa que classifique íons com base em suas massas (que usam campos eletromagnéticos), e em um detector que meça o valor de um indicador e forneça dados calculando a plenitude dos íons actuais (MORAES et al., 2013).

Após obtidos os dados de sequenciamento dos peptídeos, são comparados com um banco de dados através de softwares como Mascot ou Sequest. Nestes softwares se encontram dados de fragmentação teóricos de proteínas que permitem estimar a confiabilidade das sequencias obtidas para sua identificação (MORAES et al., 2013).

Em abelhas, estudos proteômicos com técnicas 2D-PAGE vem sendo utilizados para a investigação do desenvolvimento das colônias sob efeito de diferentes condições ambientais. Estudos recentes analisaram o proteoma do cérebro de abelhas A. mellifera (ZALUSKI et al., 2020); desenvolvimento de glândulas hipofaringeanas; e de proteínas da geleia real (ARAUJO et al., 2020).

Entretanto, estudos na área de nutrição animal, que avaliam as alterações das proteínas da geleia real em colônias que recebem suplementos nutricionais são escassos, tornando importante a análise das alterações fisiológicas e metabólicas em abelhas suplementadas com diferentes nutrientes essenciais. Dessa forma, estudos com a suplementação do mineral zinco em abelhas A. mellifera e seus reflexos no proteoma da geleia real podem contribuir para o conhecimento das reais exigências nutricionais desses insetos.

4.0 Transcriptoma

A análise do transcriptoma permite estudar o conjunto completo de transcritos de RNAs mensageiros de um determinado organismo (Passos 2014). Essa ferramenta possibilita a análise da expressão total da rede de genes envolvidos nas vias metabólicas

em determinado momento, em resposta à estímulos internos e externos, como ambiental ou nutricional do organismo modelo de estudo (GREENBAUM et al., 2001).

Em abelhas *A. mellifera* o estudo do transcriptoma tem sido realizado para elucidar diferenças na expressão gênica de abelhas infectadas por vírus transmitido pela *Varroa destructor*, bem como em descobrir quais são os genes envolvidos no comportamento higiênico contra este parasita, também utilizado para comparar rainhas e operárias de mesma subespécie e entre ovários ativados e não ativados de rainhas e operárias (LE CONTE et al., 2011; WANG et al., 2012, NIU, et al., 2014, RYABOV et al., 2014). Consequentemente, estudar o transcriptoma é essencial para interpretar os elementos funcionais do genoma de abelhas, o que acarretará na descrição de características complexas, governadas por interações de muitos genes em respostas a determinados estímulos ambientais ou nutricionais.

A área de nutrigenômica se volta ao entendimento dos efeitos de nutrientes sobre os mecanismos envolvidos na expressão gênica utilizando como ferramentas a análise do transcriptoma e proteômica. Desta forma utilizar análises atuais para o entendimento das exigências nutricionais das abelhas *Apis mellifera* se tornam necessários.

O presente trabalho tem como objetivo avaliar o efeito da suplementação de zinco (Zn) em diferentes concentrações na alimentação de abelhas *Apis mellifera* e seus reflexos no perfil metaloproteômico da geleia real e no transcriptoma das abelhas.

A presente pesquisa resultou em dois artigos que serão submetidos aos periódicos *Journal of Apicultural Research* e *BMC Genomics*, respectivamente:

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"Zinc supplementation modifies brain tissue transcriptome of *Apis mellifera* honeybees".

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CAPÍTULO 2

Organic zinc supplementation modifies the metalloproteome of royal jelly produced by *Apis mellifera*

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Short title: Organic zinc modifies the metalloproteome of royal jelly produced by *A. mellifera*.

Abstract

In this study, we analyzed *Apis mellifera* royal jelly proteome alterations induced by supplementation with different concentrations of dietary organic zinc (0, 25, 50, and 75 ppm). Proteins extracted from royal jelly were separated by electrophoresis and isoelectric focusing, and zinc-containing protein spots were identified by tandem mass spectrometry. Organic zinc-containing diets modified total royal jelly protein content and altered expression of five major royal jelly proteins (MRJP1, MRJP2, MRJP3, MRJP5, and MRJP8). While the diet with the lowest tested zinc content (Zn25) increased total royal jelly protein content and expression of MRJP1, diets with higher zinc contents (Zn50 and Zn75) decreased MRJP expression. Results suggest that dietary organic zinc content influences royal jelly quality.

Keywords: *Apis mellifera*, zinc, royal jelly, proteome.

1. INTRODUCTION

Honeybees (*Apis mellifera*) require specific nutrients to maintain their health. Nutrient deficiency increases susceptibility to damage and death due to exposure to pathogens (e.g. parasites) and/or toxins (e.g. pesticides) (Dolezal et al., 2019; Dolezal et al., 2018). Among elemental micronutrients, zinc is notable as the second most important essential micronutrient required by all living organisms, playing a critical role in various key biological processes (including regulation of cell division, protein synthesis, and DNA synthesis) (Tosi et al. 2017; Faa et al. 2008; Eyer & Dieteman, 2016.)

Approximately 40 % of proteins and enzymes require the presence of a metal ion co-factor, such as zinc, for biological functionality (Frassinetti et al. 2006; Romanowski et al., 2001): metal ions bound to enzymes act as catalysts for enzyme systems (Hilton, 1989). Within the antioxidant system, for example, zinc facilitates the function of enzymes (e.g. Cu/Zn-SOD) responsible for eliminating reactive oxygen species (ROS) (Corona & Robinson, 2006; Shaahenn & EI-Fattah, 1995), which can otherwise damage biological macromolecules (Sohal & Weindruch, 1996).

In animal nutrition, chelates (organic molecules containing bonded minerals) rather than inorganic minerals are the superior dietary form, offering absorption advantages due to decreased competition with other minerals for binding sites (Peixoto et al. 2005). However, few studies have examined the impact of such nutrients on *A. mellifera*. Under natural conditions, honeybees depend exclusively on two food sources: nectar as a source of energy and pollen as a source of proteins, vitamins, and minerals (including zinc) (Potts et al., 2016; Ghosh, 2017). Royal jelly production is directly related to the quality of pollen consumed by worker bees, but during certain periods of the year pollen sources become scarce (Lin et al., 2018). Royal jelly is produced by the glandular cephalic system (mandibular and hypopharyngeal glands) of nursing bees (Brodschneider & Crailsheim, 2010). It is composed of water, proteins, vitamins, minerals, fats, sulfur, ethereal extracts, and sucrose, and is used to feed both worker bees (during their larval stage) and the queen (throughout her lifespan) (Potts, et al., 2016).

The main constituents of royal jelly are the major royal jelly proteins (MRJPs), particularly excellent sources of protein which influence the queen's ovarian development and act as caste determinants (Alves, 2012; Uniprot, 2020). Proteomic analysis is commonly used to analyze royal jelly protein profile variations due to changing environmental, nutritional, or physiological conditions (Li et al., 2007; Kamakura, 2011; Fujita, 2012). Knowledge of

proteome (including metalloproteome) changes can provide important information contributing to honeybee nutritional management. Examples of methods used to investigate royal jelly proteome (and therefore quality) changes include protein separation techniques such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mineral characterization via mass spectrometry (MS) (Hilton, 1989; Sun et al., 2007; Xiao-Fan et al. 2005).

This study evaluated the effects of differing levels of dietary organic zinc (zinc methionine) content on *A. mellifera* royal jelly proteome composition.

2. MATERIAL AND METHODS

2.1. Location

Experiments were conducted at an apiary in the Beekeeping Production Area of the Lageado Experimental Farm, Faculty of Veterinary Medicine and Animal Science, UNESP, Botucatu, São Paulo, Brazil (22°49' S, 48°24' W). This location is at an average altitude of 623 m above sea-level, and is characterized by a humid subtropical climate.

2.2. Experimental Groups

Twenty Africanized *A. mellifera* beehives, standardized for number of breeding and feeding frames, were randomized to four groups (five hives per group): those receiving sucrose syrup (1:1 m/v sucrose:water ratio) without organic zinc supplementation (Zn0 control group) and those receiving sucrose syrup supplemented with 25, 50, or 75 ppm organic zinc (Zn25, Zn50, and Zn75 experimental groups). These values were based on the recommended 50 ppm value described by Herbert and Shimanuki (1978). After syrup preparation, organic zinc levels were confirmed by flame atomic absorption spectroscopy (FAAS), providing values as follows: 0.00 ppm (Zn0), 23.16 ppm (Zn25), 48.08 ppm (Zn50), and 74.44 ppm (Zn75). Ferrous zinc methionine (16 % zinc) was used as the organic zinc source, and syrup (with or without supplementation) was supplied to bees using a Boardman feeder (500 mL per week for one month).

2.3. Royal Jelly Collection

Royal jelly was produced by larval transfer, according to the method described by Doolittle (1899). Briefly, royal jelly was produced in a mini-hive system composed of two superposed nucleus hives separated by a screen. Queens were temporarily removed to the support hive in order to increase the need for a new queen in the original hive. Using an

appropriate brush, three day-old larvae were transferred to acrylic cups (one larva per cup) containing a drop of royal jelly diluted in water (1:1 ratio). Two bars containing 25 acrylic cups each were introduced into the queen-free hive. After 48 h, royal jelly was harvested and stored in Falcon tubes at -20°C preceding analysis, and queens were returned to their original hives.

2.4. Extraction and Precipitation of Royal Jelly Proteins

Royal jelly proteins were extracted by placing 250 mg of royal jelly into a 2 mL microcentrifuge tube, adding 500 μL of ultrapure water, vortexing, and centrifuging (5543 × g, 4 °C, 30 min). Supernatants were subjected to six further rounds of centrifugation (22,172 × g, 4 °C, 5 min) to remove all particulates. Proteins were precipitated by adding 400 μL of 80 % acetone to 100 μL of supernatant and incubating for 2 h. Proteins were pelleted by centrifugation (22,172 × g, 4 °C, 5 min) and pellets were washed three times with 200 μL of 80 % acetone. Total protein concentration was estimated using the Biuret method in conjunction with bovine serum albumin standard curves (Braga et al., 2015).

2.5. 2D-PAGE

Protein pellets were solubilized in a solution containing 7 mol L^{-1} urea, 2 mol L^{-1} thiourea, 2 % (m/v) 3-(3-chloroaminopropyl) dimethylammonium sulfate-propan-1, 0.5 % (v/v) ampholytes at pH 3-10, 0.002 % (m/v) bromophenol blue, and 2.8 mg dithiothreitol (DTT) (Braga et al., 2015; Vieira et al., 2015). Of this solution, 250 μL (containing approximately 375 μL protein) was applied to a 13 cm immobilized pH gradient (IPG) strip (pH range: 3–10) rehydrated in immobiline buffer and incubated for 12 h. The first dimension of separation, then, comprised isoelectric focusing (IEF), performed using an EttanIPGphor system ().

Strips were then sequentially submerged in two solutions (15 min each, with stirring): (1) 10 ml reducing solution (containing 6 mol L^{-1} urea, 2 % (m/v) SDS, 30 % (v/v) glycerol, 50 mmol L^{-1} Tris-HCl (pH 8.8), 0.002 % (m/v) bromophenol blue, and 2 % (m/v) DTT to maintain proteins in their reduced forms) and (2) alkylating solution (of identical composition, but replacing DTT with 2.5 % (w/v) iodoacetamide (IAA) to prevent re-oxidation of reduced protein thiol groups). Strips were applied to 12.5 % (m/v) polyacrylamide gels. Molecular weight (MW) standards of 14 to 97 kDa were added alongside each strip. Both were sealed with 0.5 % (w/v) agarose solution (Braga et al., 2015) and PAGE was performed in two phases: 100 V for 30 min followed by 250 V for 2.35 h. Proteins were fixed within the gel by exposure to 10 % (v/v) acetic acid and 40 % (v/v) ethanol for 1 h, followed by staining with colloidal Coomassie blue for 72 h.

Gels were then washed in ultrapure water, scanned, and analyzed using ImageMaster Platinum software version 7.0 () to determine the number of matched protein spots (Braga et al., 2015; Santos et al., 2015; Lima et al., 2010).

2.6. Determination of zinc presence within protein spots by FAAS

Methodology used to determine protein zinc content was adapted from Moraes et al. (2012). Briefly, gel protein spots were transferred to 5 mL digestion tubes, each containing 500 μL of ultrapure concentrated sulfuric acid and 100 μL of 30 % (m/m) hydrogen peroxide. Tubes were incubated in a digestion block until mineralization was complete (i.e. samples had become transparent). Next, acid extracts were added to 5 mL ultrapure water (18.2 M Ω cm^{-1}). Standard curves were generated by preparing an optimal range of zinc solutions with different concentrations as per manufacturer instructions (Shimadzu, 2007). Titrisol standards containing 1000 mol L^{-1} Zn were diluted in 0.10 mol L^{-1} H_2SO_4. Default operating parameters for FAAS were used, as per manufacturer instructions.

2.7. Tryptic digestion of protein spots

Each protein spot was cut from the gel, further divided into segments of approximately 1 mm^3, transferred to a 2 mL tube containing 200 μL of 5 % acetic acid, and stored at −4 °C until analysis. Gel segments were washed at least three times (30 min per cycle) using a bleach solution containing 50 % (v/v) acetonitrile (CH_3CN) and 25 mmol L^{-1} ammonium bicarbonate (NH_4HCO_3) and were dehydrated twice (10 min per cycle) using 100 % CH_3CN. After the second dehydration cycle, CH_3CN was removed, and remaining residue was allowed to evaporate at room temperature.

Treated spots were reduced using 20 mmol L^{-1} DTT in 50 mmol L^{-1} NH_4HCO_3 for 40 min at 56 °C. Next, the solution was replaced with fresh solution containing mmol L^{-1} IAA in 50 mmol L^{-1} NH_4HCO_3, followed by incubation for 30 min at room temperature, in the dark. The solution was removed and fragments were washed using 25 mol L^{-1} NH_4HCO_3 and dehydrated using 100 % CH_3CN. After dehydration, CH_3CN was removed, and remaining residue was allowed to evaporate at room temperature.

For protein digestion, gel fragments were rehydrated for 15 min in a 15 μL solution containing 150 ng trypsin (Trypsin GoldMass Spectrometry, Promega, Madison, WI, USA) in 25 mmol L^{-1} NH_4HCO_3. Next, 50 μL of 25 mM NH_4HCO_3 was added for complete coverage of spots, prior to incubation for 14 h at 37 °C. Tryptic digestion was halted by acidification using 15 μL of blocking solution containing 50 % (v/v) CH_3CN and 5 % (v/v) formic acid.

Peptides were extracted from acrylamide particles via two washes (15 min per cycle) using elution solution A (containing 50 % (v/v) CH_3CN and 1 % (v/v) formic acid), one wash (15 min) using elution solution B (containing 60 % methanol and 1 % (v/v) formic acid), and two washes using 100 % CH_3CN at 45 °C and with sonication at 40 kHz and 30 W (Ultracleaner 1600A, Unique, Brazil). Extracted peptides were vacuum-dried (Eppendorf, Hamburg, Germany) for 6-8 h at 25 °C, and stored at −20 °C until analysis (Shevchenko, 2007).

2.8. Identification of proteins by ultraperformance liquid chromatography-coupled electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS)

Aliquots of peptide solutions were injected onto a nano Acquity UPLC column (Waters, Milford, MA, USA) for separation. Eluting peptides were ionized for entry into a mass spectrometer employing a Xevo G2 hybrid Q-TOF mass analyzer (Waters, Milford, MA, USA). Mass spectra were acquired and the raw data were submitted for identification, using an *A. mellifera* protein reference database downloaded from UniProt.

2.9. Data Analysis

Gel images were analyzed using ImageMaster 2D Platinum version 7.0 software (GE Healthcare), which facilitates identification of equivalent spots between gels. Three replicate gels were produced per experimental group. During image analysis, spots were compared for distribution, volume, relative intensity, isoelectric point, and molecular mass using linear regression GE (Moraes et al..2012).

As described above, protein group identities were inferred from tryptic peptide mass spectra using a reference proteome downloaded from UniProt. Additionally, relative quantitation of protein groups was performed using this same software. The National Center for Biotechnology Information (NCBI)'s online Blast2GO algorithm (www.blast2go.com, 2020) was used to infer protein functional annotation based on sequence similarity to proteins with known function (Conesa et al., 2005). Between-group quantitative differences were evaluated using analysis of variance (ANOVA), followed by a Tukey test to determine significance of differences (significance threshold $p < 0.05$). Each experimental group's protein quantities were compared to the those of the control. All statistical analyses were performed using SAS statistical software version 8 (Zar, 2010).

3. RESULTS

3.1. Royal jelly protein content

A significantly higher total royal jelly protein content was noted for colonies that received diets supplemented with the lowest level of zinc (Zn25), relative to colonies receiving diets supplemented with higher levels of zinc (Zn50 and Zn75) ($p < 0.05$) (Table 1).

Table 1 Total royal jelly protein concentration (g L^{-1})

Treatment	Mean ± SD
Zn0	13.6 ± 0.17b
Zn25	15.4 ± 0.75a
Zn50	13 ± 0.51b
Zn75	13.6 ± 0.13b

Zn0 no added Zn, **Zn25** 25 ppm added Zn, **Zn50** 50 ppm added Zn, and **Zn75** 75 ppm added Zn. Letters indicate statistical difference between experimental groups ($p < 0.05$).

Relatively higher numbers of zinc-containing protein spots were noted for colonies that received no or the lowest level of dietary zinc supplementation (Zn0 and Zn25), whereas colonies that received higher levels of dietary zinc supplementation (Zn50 and Zn75) exhibited relatively fewer zinc-containing protein spots (Fig. 1). Indeed, the number of zinc-containing proteins spots was significantly higher for the Zn25 (relative to the Zn50 and Zn75) groups ($p < 0.05$).

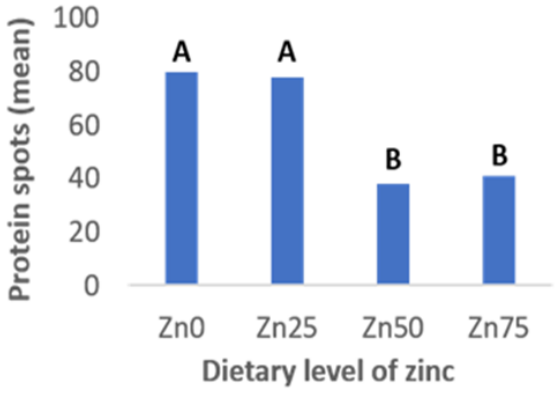


Figure 1 Number of royal jelly zinc-containing protein spots detectable during image analysis. **Zn0** no added Zn, **Zn25** 25 ppm added Zn, **Zn50** 50 ppm added Zn, and **Zn75** 75 ppm added Zn. Letters indicate statistical difference between experimental groups (p<0,05).

3.2. Metalloprotein characterization and quantitation

Gene ontology (GO) annotation of proteins identified from zinc-containing spots demonstrated that zinc-containing metalloproteins are involved in crucial *A. mellifera* physiological functions, including metabolism and defense (Fig.s 2 and 3).

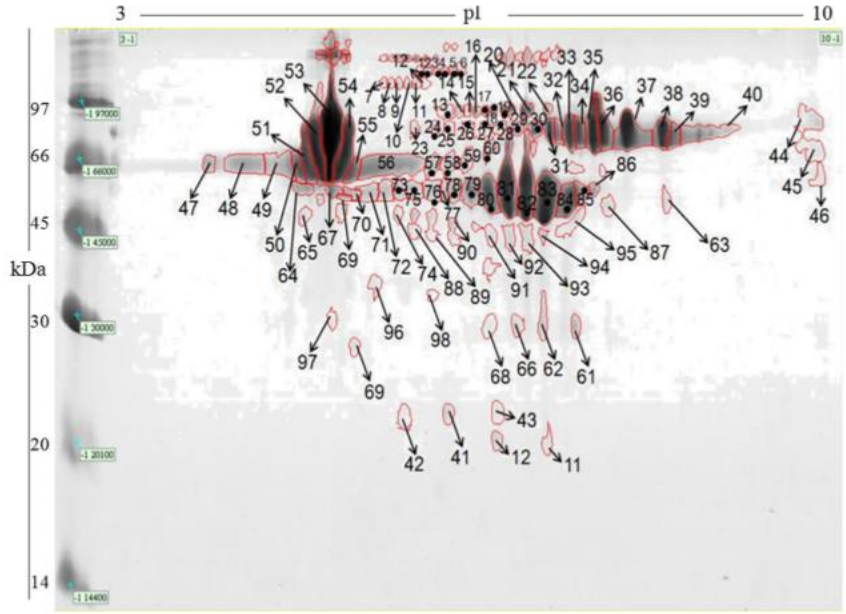
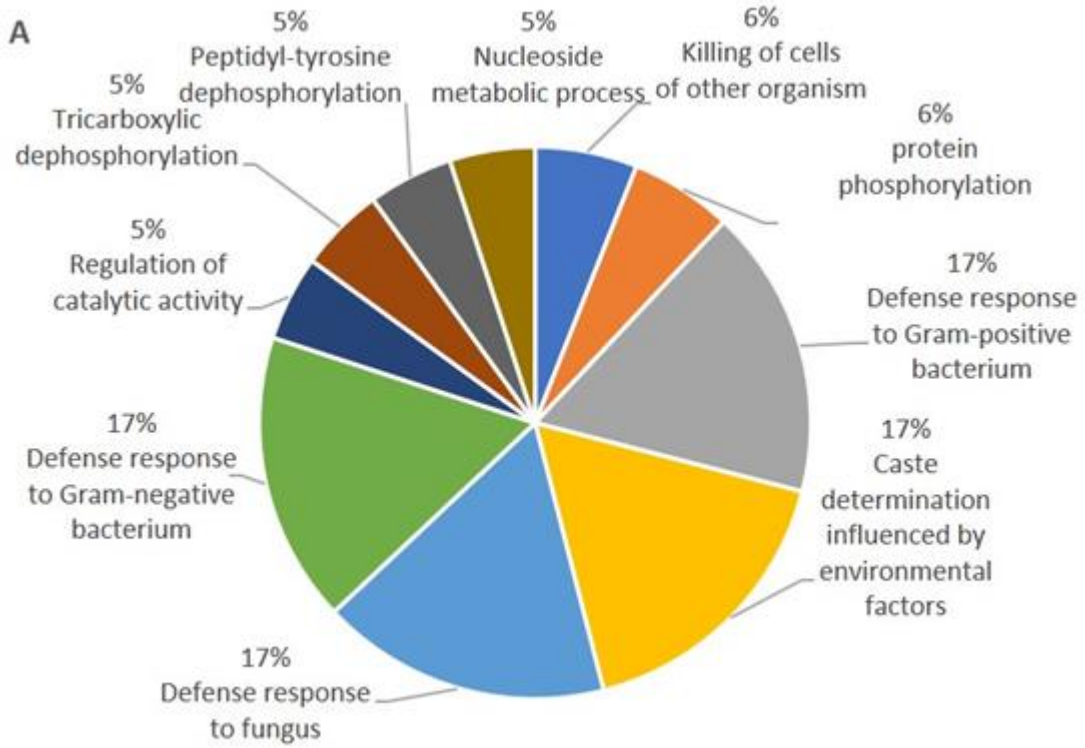
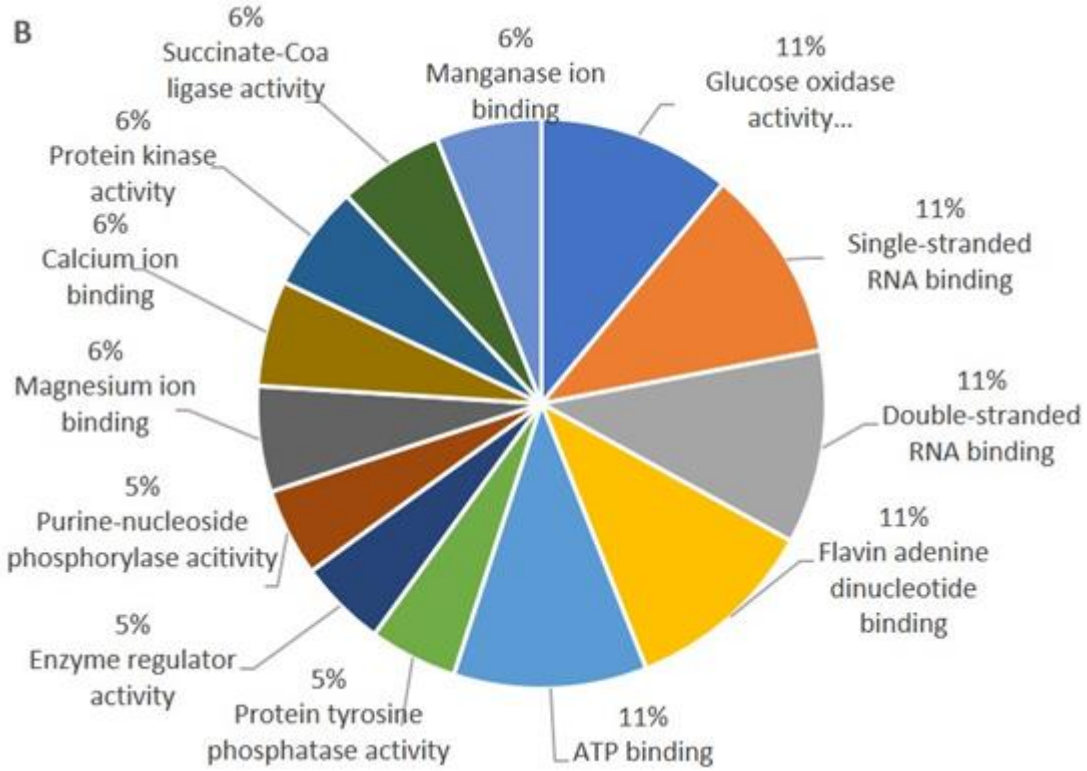


Figure 2 Representative image of polyacrylamide gel protein spots after 2D-PAGE. All numbered spots were subjected to UPLC-ESI-MS/MS analysis.

Figure 3 Metalloprotein GO Annotations: biological process (**A**) and molecular function (**B**).





When comparing experimental group levels of each royal jelly metalloprotein to those in the control group, the Zn25 group exhibited 19 significantly differentially-expressed spots (11

up-regulated, 8 down-regulated), the Zn50 group exhibited 25 differentially-expressed spots (10 up-regulated, 15 down-regulated), and the Zn75 group exhibited 15 differentially-expressed spots (7 up-regulated, 8 down-regulated) (Table 2).

Table 2 Differential royal jelly metalloprotein expression induced by dietary supplementation with differential quantities of organic zinc.

		Zn25		Zn50		Zn75	
MJRP group ID	**Biological process/function**	**Up-regulated**	**Down-regulated**	**Up-regulated**	**Down-regulated**	**Up-regulated**	**Down-regulated**
MJRP1	Caste determination, defense responses	6	2	3	6	2	4
MJRP2	May play an important role in honeybee nutrition	2	3	3	3	2	-
MJRP3	May play an important role in honeybee nutrition	-	3	2	4	2	3
MJRP5	May play an important role in honeybee nutrition	-	-	-	1	1	1
MJRP8	-	3	-	2	1	-	-
Total		**11**	**8**	**10**	**15**	**7**	**8**

Metalloprotein up- and down-regulation is reported relative to the control group.

4. DISCUSSION

Organic zinc supplementation influenced both royal jelly total protein content and the number of zinc-containing proteins. Given that a significantly higher level of total protein as well as number of zinc-containing protein spots was observed in the Zn25 group, either low-level organic zinc supplementation is beneficial, or high-level supplementation is detrimental to *A. mellifera* metabolism.

Different levels of dietary organic zinc supplementation appear to directly modulate expression of proteins in royal jelly produced by *A. mellifera*. Royal jelly produced by the Zn25 group exhibited a higher overall protein content (relative to both the control and other experimental groups) and a higher number of up-regulated metalloprotein spots (relative to the other experimental groups: Zn50 and Zn75).

In all experimental groups, the protein group exhibiting the greatest change in expression was major royal jelly protein 1 (MRJP1_APIME). This group of proteins is involved in essential biological processes, including caste differentiation and defense against fungi and bacteria. For example, MRJP1 proteins are associated with the differentiation of larvae into queens via an epidermal growth factor receptor-mediated signaling mechanism (Hartfelder et al., 2006). Specifically, MJRP1 proteins are linked to increased body size via activation of p70 S6 kinase, ovarian development via mitotic regulation, an increase in vitellogenin (the main reproductive protein of insects) expression, and the production of juvenile hormones related to the external morphology of female bees (Hartfelder et al., 2006).

Zinc is a component of many classes of metalloproteins and metalloenzymes (Eisler, et al., 1993). The amount of zinc in the bee diet can influence several metabolic processes, since zinc ions transcytose across midgut epithelia for transport through hemolymph by vitellogenin (Cousins et al., 2006; Barbehenn & Martin, 1992; Falchuk, 1998). Our results suggest that supplementation with 25 ppm of organic zinc promoted a significant increase in metalloprotein expression.

In contrast, Zhang et al. (2015) has suggested that 30 ppm of *in*organic zinc enhances bee antioxidant activity (enzymatic activity of Cu/Zn-SOD) and longevity, and that 60 to 70 ppm optimizes royal jelly production. However, source of the mineral is indeed a critical determinant of supplementation outcome. Minerals provided within organic sources react differently in the body than their inorganic forms, exhibiting improved absorption due to less competition for binding sites with other minerals (Peixoto et al., 2005). This likely explains the discrepancy between our results (using an organic zinc source) and those of Zhang et al. (using an inorganic zinc source). Contrary to the Zn25 group, higher levels of zinc supplementation (Zn50 and Zn75 groups) negatively impacted royal jelly protein content, including expression of MJRP class proteins, which play an important role in bee development. Indeed, zinc overconsumption is known to have toxic effects (e.g. increased ROS production) in many animals (Eisler, 1993). However, zinc-dependent Cu/ZN-SOD enzymes help *decrease* the accumulation of free radicals (Corona, 2006). Therefore, an optimal dietary zinc concentration is required to appropriately stimulate antioxidant activities that assist in the maintenance of *A. mellifera* colony health.

There is a dearth of published research regarding *A. mellifera* nutrigenomics, including recommendations for ideal levels of mineral supplementation. Therefore, this study

characterized impact on the royal jelly metalloproteome of dietary organic zinc supplementation at different levels, including impact on MJRP expression.

5. Conclusions

Our results suggest that dietary organic zinc supplementation at 25 ppm increases the protein content of royal jelly, including expression of zinc-dependent metalloproteins involved in key molecular and biological processes. Further studies investigating the impact of lower doses of organic zinc supplementation are warranted.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Supplementary Material

Proteins of the group "major royal jelly proteins" (MRJPs)							
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 1 (MRJP1_APIME)							
1	-0.0465	+14.217	Ø	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 another organism.
22	Ø	Ø	+0.1495	203.3688		8.56	
29	-0.0525	0.2698	Ø	348.1182		2.55	
37	46.2366	46.5031	-47.536	1963.112		45.6	
38	0.8843	-3.5824	-1.3673	12041.74		49.77	
39	0.6194	-0.4177	-0.5784	11584.09		56.25	
40	+0.1774	-0.2985	-0.2141	8034.015		51.16	
43	+6.2887	1.5866	+2.6754	3618.422		33.33	
46	+0.3534	+4.2325	Ø	231.0561		3.01	
51	Ø	-0.0630	Ø	1076.779		2.55	
54	+0.2888	Ø	Ø	5778.884		45.6	
57	-0.5101	-1.6702	Ø	111.2232		3.01	
58	+1.7484	+4.2325	0.5714	2390.795		31.25	
64	+7.7446	-1.2508	Ø	348.0592		8.1	
Major royal jelly protein 2 (MRJP2_APIME)							
22	Ø	Ø	+0.1495	70.2529	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 honeybee larva. The royal jelly determines the development of the young larvae and is responsible for the high reproductive ability of the honeybee queen.
31	-1.1034	-1.9810	Ø	321.1932		10.4	
34	-2.7349	-2.1384	0.8310	7363.174		50.44	
40	+0.1774	+0.2985	+0.2141	395.2112		18.58	
58	+1.7484	+4.2325	0.5714	101.0753		2.43	
59	0.6933	+5.0882	1.3717	311.52582.43		2.43	
64	7.7446	-1.2508	Ø	1431.684		2.43	
67	-0.2915	-0.3314	Ø	915.2661		2.43	
Major royal jelly protein 3 (MRJP3_APIME)							
22	Ø	Ø	+0.1495	338.3209	6.47/61.662	15.07	May play an important role in honeybee nutrition. It is found in the royal jelly which is the food of the queen honeybee larva. The royal jelly determines the development of the young larvae and is responsible for the high reproductive ability of the honeybee queen.
31	1.1034	+1.9810	Ø	166.6245		5.88	
38	0.8843	+3.5824	+1.3673	40.8037		5.88	
39	0.6194	-0.4177	-0.5784	729.9177		29.41	
40	-0.1774	-0.2985	-0.2141	673.7283		18.2	
64	7.7446	-1.2508	Ø	8065.417		47.79	
66	-1.0969	5.0882	-0.2094	4662.946		41.36	
67	-0.2915	-0.3314	Ø	3666.14		40.26	
Major royal jelly protein 5 (MRJP5_APIME)							
33	3.2460	-1.3793	1.2058	44.4162	5.95/70.236	2.02	May play an important role in honeybee nutrition. It is found in the royal jelly which is the food of the queen honeybee larva. The royal jelly determines the development of the young larvae and is responsible for the high reproductive ability of the honeybee queen.
39	0.6194	0.4177	-0.5784	2645.292		1.84	
43	6.2887	1.5866	+2.6754	910.464		1.84	
Major royal jelly protein 8 (Q6TGR0_APIME)							
24	+0.5253	0.0820	15.902	7.1	6.00/46.956	1.44	-
25	+0.5485	0.2934	0.0329	5.5901		1.44	
58	+17.484	+4.2325	0.5714	13.8635		1.44	
59	0.6933	+5.0882	13.717	45.1301		1.44	
64	7.7446	-1.2508	Ø	109.8327		1.44	

Table 1 Identification of protein spots with different expression (ANOVA; P<0.05) in the royal jelly of feeding bees fed 25 ppm zn (Zn25), 50 ppm zn (Zn50), and 75 ppm zn (Zn75). Expression analysis of the spots was performed using the ImageMaster Platinum program, with reference to the proteomics of non-mineral zinc supplemented bees (Zn0) with the identification of proteins by ESI-MS/MS. Positive (+) values show upregulated protein spots, and negative values (−) show downregulated protein spots 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.

CAPÍTULO 3

Zinc supplementation modifies brain tissue transcriptome of *Apis mellifera* honeybees

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Abstract

Background: Bees are the most important group of pollinators worldwide and their populations are declining. In natural conditions, *Apis mellifera* depends exclusively on food from the field to meet its physiological demands. In the period of scarcity, available resources are insufficient and artificial supplementation becomes essential for maintaining the levels of vitamins, proteins, carbohydrates, and minerals of colonies. Among these minerals, zinc is essential in all living systems, particularly for the regulation of cell division and protein synthesis, and is a component of more than 200 metalloenzymes.

Results: The total RNA extracted from the brain tissue of nurse bees exposed to different sources and concentrations of zinc was sequenced. A total of 1,172 genes in the treatment that received an inorganic source of zinc and 502 genes that received an organic source of zinc were found to be differentially expressed among the control group. Gene ontology enrichment showed that zinc can modulate important biological processes such as nutrient metabolism and the molting process.

Conclusions: Our results indicate that zinc supplementation modulates the expression of many differentially expressed genes and plays an important role in the development of *Apis mellifera* bees. All the information obtained in this study can contribute to future research in the field of bee nutrigenomics.

Keywords: *Apis mellifera,* Zinc, Gene expression, Nutrigenomics

Background

Bees are the most important group of pollinators in the world that visit 90% of the major 107 global crop types and assist the maintenance of ecosystems [1]. *Apis mellifera* honeybees are the most commonly managed species, which provide high-value pollination services for agricultural crops [1]. Recent declines in honeybees population have been attributed to multiple interacting stressors, of which, the most important are nutrition deficits, pesticides, parasites, and pathogens [2].

Under natural conditions, *Apis mellifera* bees depend exclusively on two food sources to supply colonies: nectar as a source of energy and pollen as a source of protein, vitamins, and minerals [3,4]. Minerals are needed to maintain life because they play significant roles as structural components and enzymatic cofactors [5]. Among the essential minerals, some are distinct for participating in important metabolic pathways, such as zinc [6].

Zinc is the second most essential element in all living systems and is essential for the regulation of cell division, protein synthesis, and DNA [6,7,8]. Zinc ions can penetrate the peritrophic membrane and midgut epithelial cells, and transporters for delivering zinc ions can be influenced by dietary Zn levels [7]. This microelement is transferred to the hemolymph, where vitellogenin acts as the main Zn transporter [8,9]. Zinc is a component of more than 200 metalloenzymes and other metabolic compounds that modulate biochemical processes which involve the maintenance of cell membrane integrity, cellular respiration and reproduction, and other essential functions such as binding nucleic acids as a zinc finger complex [10].

Zinc cannot be stored in the body [11] and requires regular dietary intake to meet physiological needs. Thus, zinc is routinely supplemented in human and livestock foods and feeds for normal physiological functions as well as to meet daily requirements [12]. Zinc is commonly supplemented in two forms: organic form (methionine) and inorganic form (sulfate); minerals linked to organic molecules called chelates have advantages over the inorganic form,

with greater absorption and less competition for binding sites with other minerals [13]. However, few studies have clarified the nutritional requirements of *Apis mellifera* bees.

The advent of next-generation sequencing (NGS) technology has revolutionized the way biological research is conducted [14]; in this study, RNA-seq was used to investigate the effects of zinc supplementation at different dosages and sources on the transcriptome profiles of *A. mellifera* bees.

Results and Discussion

Differential gene expression (DGE) library sequencing

DGE tag libraries were constructed and sequenced using total RNA extracted from the brain tissue of *A. mellifera* nursing bees. For each library, brain tissue was dissected from five worker bees and pooled as a sample to construct the library. The sequencing results showed that the four biological replicates of each sample had high reproducibility, suggesting the high reliability of the sequencing results (Table 1).

Table 1 Statistics of differential gene expression sequencing

Sample	Alignment_not_unique	Ambiguous	Feature	No_feature	Not_aligned	Too_low_aQual
Zn0-1	0% (0)	0,8%	60.9%	7.9%	1%	29.4%
Zn0-2	0% (0)	0.3%	24%	2.5%	43.3%	29.9%
Zn0-3	0% (0)	0.8%	69.9%	4.5%	1.1%	23.7%
Zn0-4	0% (0)	1.1%	86.9%	7.9%	1%	3.1%
ZnI25-1	0% (0)	0.8%	67%	6.2%	1%	25%
ZnI25-2	0% (0)	1.1%	80.3%	11.1%	1.4%	6.1%
ZnI25-3	0% (0)	1%	82.2%	8.6%	3.2%	5%
ZnI25-4	0% (0)	1%	79.8%	11.9%	2.1%	5.2%
ZnI50-1	0% (0)	0.9%	79.5%	4.6%	1%	14%
ZnI50-2	0% (0)	1%	88.5%	4.6%	0.6%	5.3%
ZnI50-3	0% (0)	0.9%	85.6%	6.4%	1.5%	5.5%
ZnI50-4	0% (0)	0.9%	83.1%	7.6%	0.8%	7.5%
ZnI75-1	0% (0)	1%	85.1%	5.4%	0.8%	7.8%
ZnI75-2	0% (0)	0.9%	77.9%	3.6%	11.4%	6%
ZnI75-4	0% (0)	0.8%	78.8%	5.9%	8%	6.4%
ZnO25-1	0% (0)	0.8%	75.5%	4.4%	0.7%	18.6%
ZnO25-2	0% (0)	0.8%	77.6%	5%	2.8%	14.7%
ZnO25-3	0% (0)	0.7%	82.6%	4.2%	1.2%	11.6%
ZnO25-4	0% (0)	1%	75.3%	3.9%	0.9%	17.3%
ZnO50-1	0% (0)	1%	75.9%	4.1%	0,9%	17.8%
ZnO50-2	0% (0)	1.2%	85.1%	7%	9.4%	6.1%
ZnO50-3	0% (0)	0.7%	86.5%	4.7%	1.8%	6.6%
ZnO50-4	0% (0)	1.1%	68.7%	10.6%	2.3%	10.5%
ZnO75-1	0% (0)	1%	85.2%	6%	1.4%	5.8%
ZnO75-2	0% (0)	0.9%	83%	7.7%	1.6%	6%
ZnO75-3	0% (0)	0.9%	80.8%	9.1%	2.3%	7.8%
ZnO75-4	0% (0)	0.9%	80.9%	4.1%	1.6%	12.6%

Analysis of gene expression of bees supplemented with different sources of zinc

After supplementing the bees with different concentrations of inorganic zinc, the treatments showed 1,172 differentially expressed genes when compared to the control, with the ZnI50 group having the highest number of upregulated genes and the Zni25 and Zni75 groups with the largest number of downregulated genes. Of these differentially expressed genes, 64, 116, and 657 were exclusively expressed in the ZnI25, ZnI50, and ZnI75 groups, respectively, and 74 genes were expressed for the three treatments, with the treatment with 75 ppm having a higher number of modulated genes (Figure 1). As we increased the dose of inorganic zinc in the diet, gene expression also increased. However, as shown in figure 2, the medium doses containing inorganic zinc (50 ppm) showed a higher number of upregulated genes compared to the downregulated ones, unlike the other dosages (25 and 75 ppm), which showed a lower number of upregulated genes.

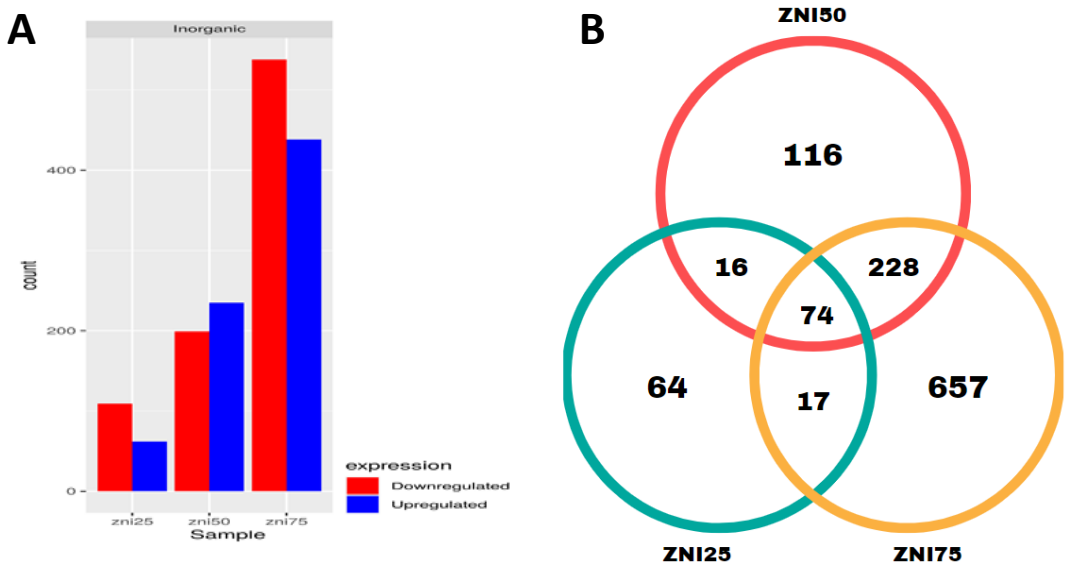

Figure 1 Differentially expressed genes (DEGs) between experimental groups (ZnI25, ZnI50, and ZnI75) compared to control group Zn0. (A) Histogram of DEGs between experimental

groups supplemented with inorganic source of zinc at each concentration of zinc (25 ppm, 50 ppm, and 75ppm). (B) Venn diagram of DEGs between experimental groups supplemented with inorganic source of zinc at each concentration of zinc.

Regarding the organic zinc supplementation, the treatments showed 502 genes that were differentially expressed when compared to the control, with the ZnO75 group having the highest number of upregulated genes and the ZnO25 and ZnO50 groups with the highest number of downregulated genes. Of these genes, 288, 16, and 57 genes were expressed exclusively in the ZnO25, ZnO50, and ZnO75 groups, respectively, and 48 genes were expressed in the three treatments, with the treatment with 25 ppm having a higher number of modulated genes (Figure 2). Contrary to what was observed with inorganic zinc, the treatment with a higher dose of organic zinc in the supplementation of *A. mellifera* bees had a smaller number of modulated genes.

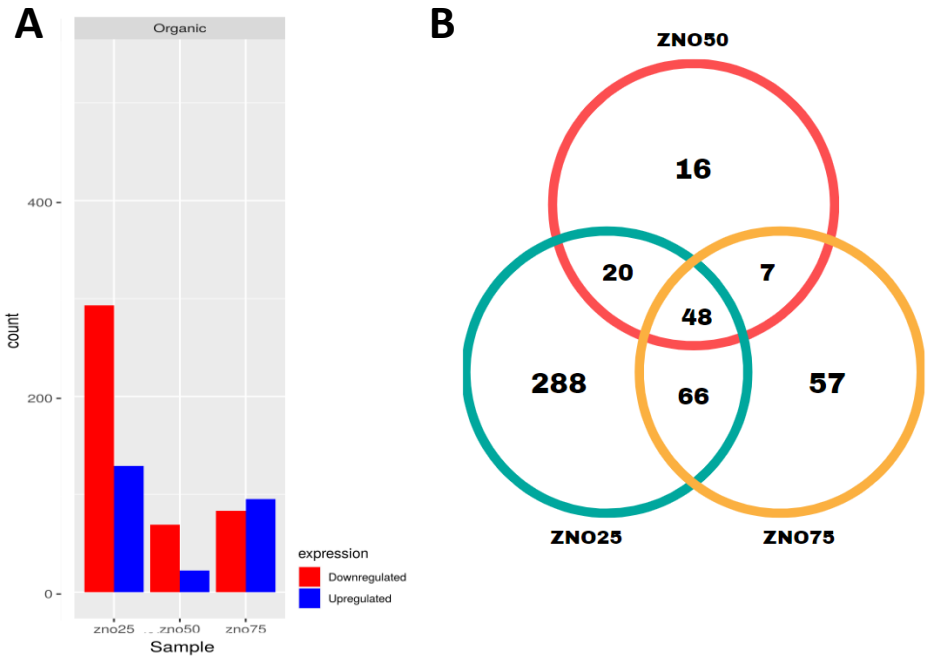

Figure 2. Differentially expressed genes (DEGs) between experimental groups (ZnO25, ZnO50, and ZnO75) compared to control group Zn0. (A) Histogram of DEGs between

experimental groups supplemented with organic source of zinc at each concentration of zinc (25 ppm, 50 ppm, and 75ppm). (B) Venn diagram of DEGs between experimental groups supplemented with inorganic source of zinc at each concentration of zinc.

Differentially expressed genes (DEGs) between treatments supplemented with organic and inorganic sources of zinc

Based on a comparison of treatments supplemented with the same concentrations of zinc, but with different sources (organic vs. inorganic), we found that organic zinc showed higher gene expression only in the lowest concentration of zinc supplementation (25 ppm). In the other experimental groups (50 and 75 ppm), inorganic zinc showed higher gene expression when compared to treatments supplemented with zinc from organic sources (Fig. 3). In addition, the results suggest that an organic source of zinc at a lower concentration (25 ppm) can increase the gene expression of *A. mellifera*. Contrasting this, for inorganic zinc, the highest concentration (75 ppm) can increase the gene expression of honeybees.

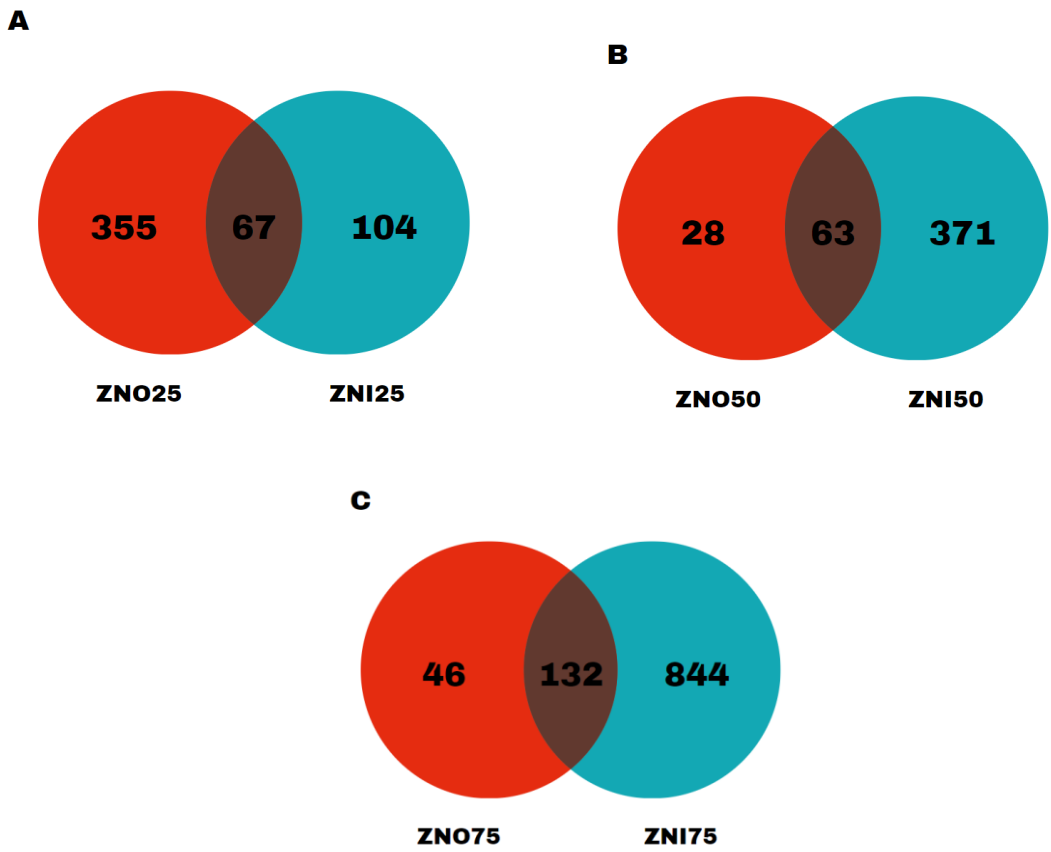

Figure 3 Venn diagrams of differentially expressed genes (DEGs) between experimental groups supplemented with inorganic and organic source of zinc at each concentration of zinc. A) Venn diagram of DEGs between experimental groups ZnO25 and ZnI25 compared to control group Zn0. B) Venn diagram of DEGs between experimental groups ZnO50 and ZnI50 compared to control group Zn0. C) Venn diagram of DEGs between experimental groups ZnO75 and ZnI75 compared to control group Zn0.

Gene Ontology enrichment analysis

In gene ontology (GO) analysis, when we compared the intersection between genes differentially expressed for the two sources of zinc (organic and inorganic), we observed that both sources stimulated genes related to important biological processes, such as chitin metabolism, amino acids, and amino glycans, as well as structural components of *A. mellifera* bee chitin (Fig. 4).

Zinc is an essential mineral for all living beings [8], and in insects, it can be related to body growth. For insects to exchange their chitin exoskeleton and complete their development, the action of chitinase enzymes is necessary, whose function is to digest the polysaccharides that form chitin [15,16,17]. During the metamorphosis process, the chitinase BmCHT5 is expressed in *Bombyx mori* [18] and when a zinc finger element is

present (BR-C Z4), the expression of this enzyme is improved. Therefore, the amount of zinc absorbed in bee nutrition can influence the action of these enzymes and, consequently, the process of molting these insects.

According to Keilling [19], zinc is a structural and catalytic component of hundreds of classes of enzymes and is highly related to the metabolism of nucleic acids and carbohydrates. In addition to affecting the functioning of digestive enzymes in rapidly growing tissues, zinc deficiency dramatically decreases the synthesis of RNA, DNA, and proteins, thereby decreasing the rate of mitosis and compromising tissue growth and recovery [11].

A. mellifera bees depend exclusively on two sources of nutrients, pollen and nectar. Pollen is composed of starch, vitamins, and minerals and depends on the action of amylase enzymes to be digested. Among these nutrients, zinc is essential for the regulation of amylase enzymes, and its function has been described in several animal species, such as pigs, rats, and chickens [20].

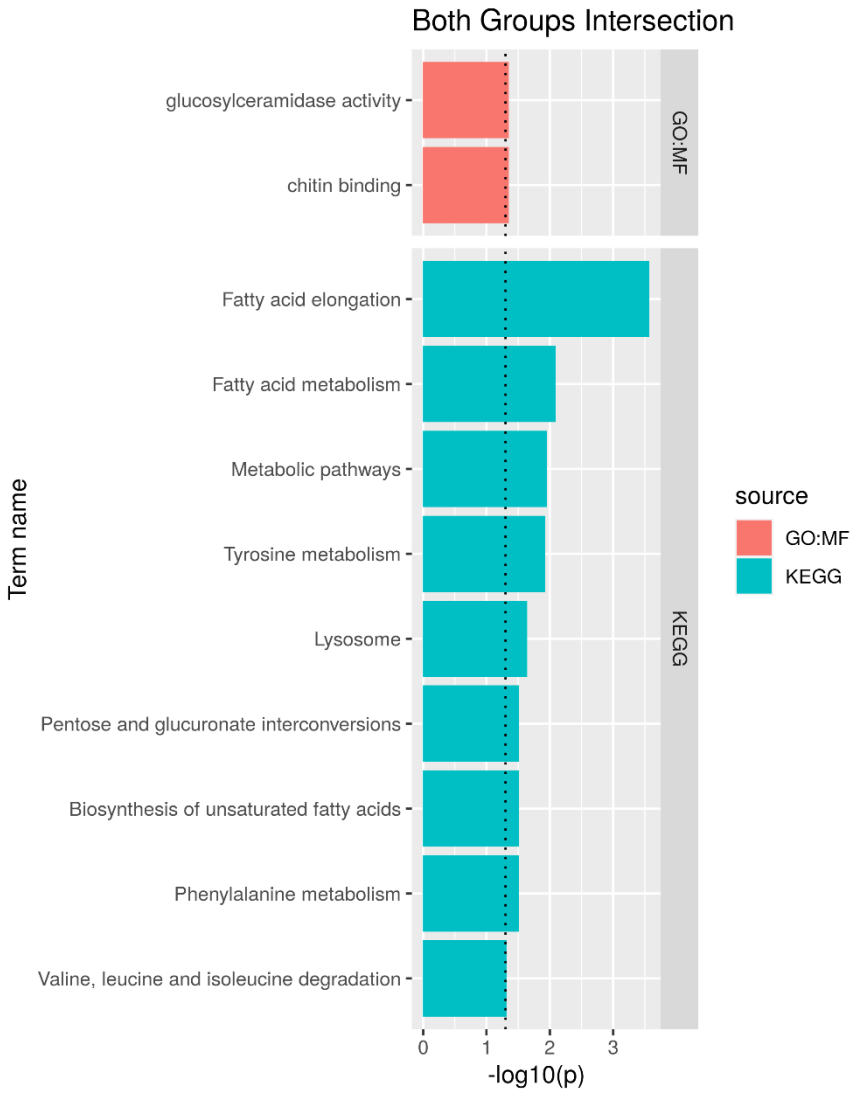

Figure 4 Gene ontology analysis of both experimental group's intersection. Red color indicates biological processes and blue color indicates KEGG.

Inorganic zinc treatments showed 1,172 differentially expressed genes, enabling the GO analysis. Our results suggest that the sources of inorganic zinc enriched the pathways related to important biological processes, such as drug and nutrient metabolism, cuticle development and metabolism, and processes related to arthropod ecdysis (Fig. 5).

Among the altered gene pathways, detoxification seems to be related to zinc supplementation. *A. mellifera* bees depend on a set of enzymes to metabolize drugs and pesticides, including cytochrome P450 monooxygenases [21]. Cytochrome P450 plays a role in the detoxification of phytochemicals present in the food consumed by bees [22,23,24]. Our

results suggest that zinc participates in the detoxification process and may modulate the action of monooxygenase enzymes in bees.

We observed the modulation of genes related to the body development processes of bees. Hiki [25] analyzed the crustacean transcriptome and reported that exposure to inorganic zinc (zinc sulfate) dosages influenced genes related to the exoskeleton, thereby inhibiting the molting process in these arthropods.

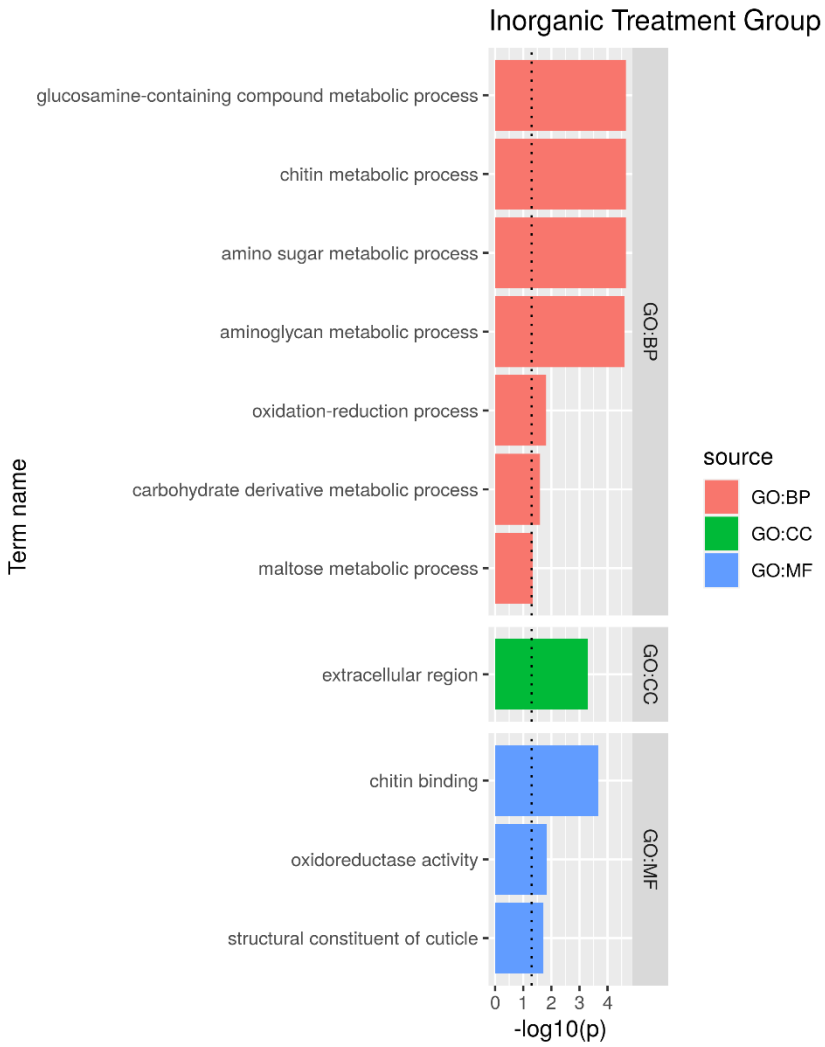

Figure 5 Gene ontology analysis of the experimental groups supplemented with inorganic source of zinc (ZnI25, ZnI50, and ZnI75). Red color indicates biological processes, blue color indicates molecular functions, and green color indicates cellular components.

For organic zinc treatments, we identified 502 differentially expressed genes, enabling the GO analysis. Our results suggest that the sources of organic zinc enriched the pathways related to important biological processes, such as nutrient metabolism and cuticle development (Fig. 6).

According to the GO analysis, organic zinc supplementation influenced specific pathways related to lipid metabolism. Organic zinc sources altered gene pathways related to the enzyme 3-hydroxyacyl-CoA dehydratase (DEH) activity, lysosome activity, and chitin binding. DEH is a key member of lipid metabolism [26] involved in very long chain fatty acid synthesis, which interacts with several elongases and plays an essential role during development, differentiation, and maintenance of a number of tissue types [27].

In insects such as Musca sp., a few lysosomes may occur and contain minerals such as Ca, Fe, Cu, P, and Zn with functions related to mineral detoxification. Insects deprived of organs with both spherocrystals and lysosomes are less able to resist mineral contamination [28]. According to the GO results, organic zinc supplementation may increase the activity of lysosomes, possibly allowing it to store the amount of zinc absorbed.

As observed in treatments that received zinc from an inorganic source, organic zinc also modulated the expression of genes related to chitin binding, suggesting a relationship between the process of ecdysis and the mineral zinc absorbed by *A. mellifera* honeybees.

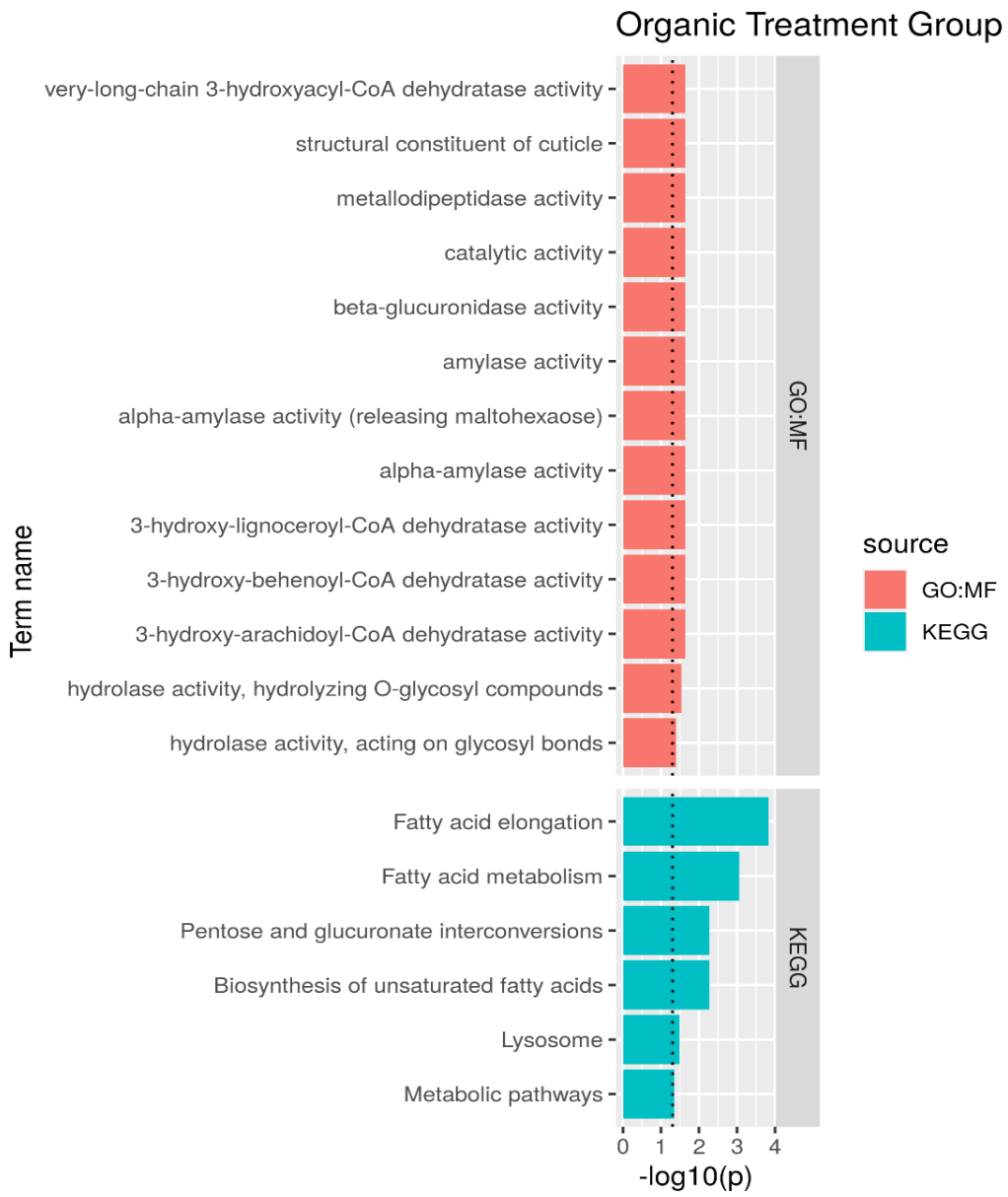

Figure 6 Gene ontology analysis of the experimental groups supplemented with organic source of zinc (ZnO25, ZnO50, and ZnO75). Red color indicates biological processes, i.e., molecular functions and the blue color indicates KEGG.

Heat map of DGE from experimental groups

The heatmap graph (Fig. 7) shows that the colonies that received dosages of inorganic zinc modulated specific genes in the control group. Among these genes, Apid1 and Burs were positively modulated.

In the figure, ‘Apid1’ refers to the apidaecin1 gene, characterized by Casteel [29], which is related to a group of antibacterial peptides, and the main humoral components of hemolymph are induced in infections caused by bacteria. ‘Burs’ refers to the Bursicon gene, a neurohumoral agent responsible for cuticle pigmentation and wing expansion during insect metamorphosis [30]. Our transcriptome analysis indicated that zinc from inorganic sources positively modulated genes related to immunity and body development processes in *Apis mellifera* bees.

Among the downregulated genes previously studied, ATP5G2, Cpap3-d, Apd-1, Apd-2, Apd-3, TpnCIIb, and CPR27, the genes of the Apd family were characterized by Kucharski [31], whose function is related to the production of cuticle proteins in *A. mellifera*. The Apd-1 gene can interfere with cuticular maturation in adults, Apd-2 is found in the internal cuticles (stomach and trachea), and Apd-3 is more widely expressed, which is related to cuticles and not pigmented.

ATP5G2 (NCBI) is related to the synthesis of ATP by mitochondria. TpnCIIb participates in the synthesis of troponin C, which is responsible for the muscle contraction mechanism [32] and CPR27 (NCBI) is a cuticular protein that is possibly related to the exoskeleton of these insects.

Concerning the referred biological processes, we observed that the inorganic zinc measurements mainly altered genes related to the arthropod body development process. As previously reported by Hiki [25], in crustaceans, zinc may have a direct relationship with the inhibition of the molting process. The process of energy synthesis (ATP) and muscle contraction mediated by troponin C also seems to be related, since at this stage the arthropod needs a large amount of energy to rupture the exoskeleton and undergoes its metamorphosis.

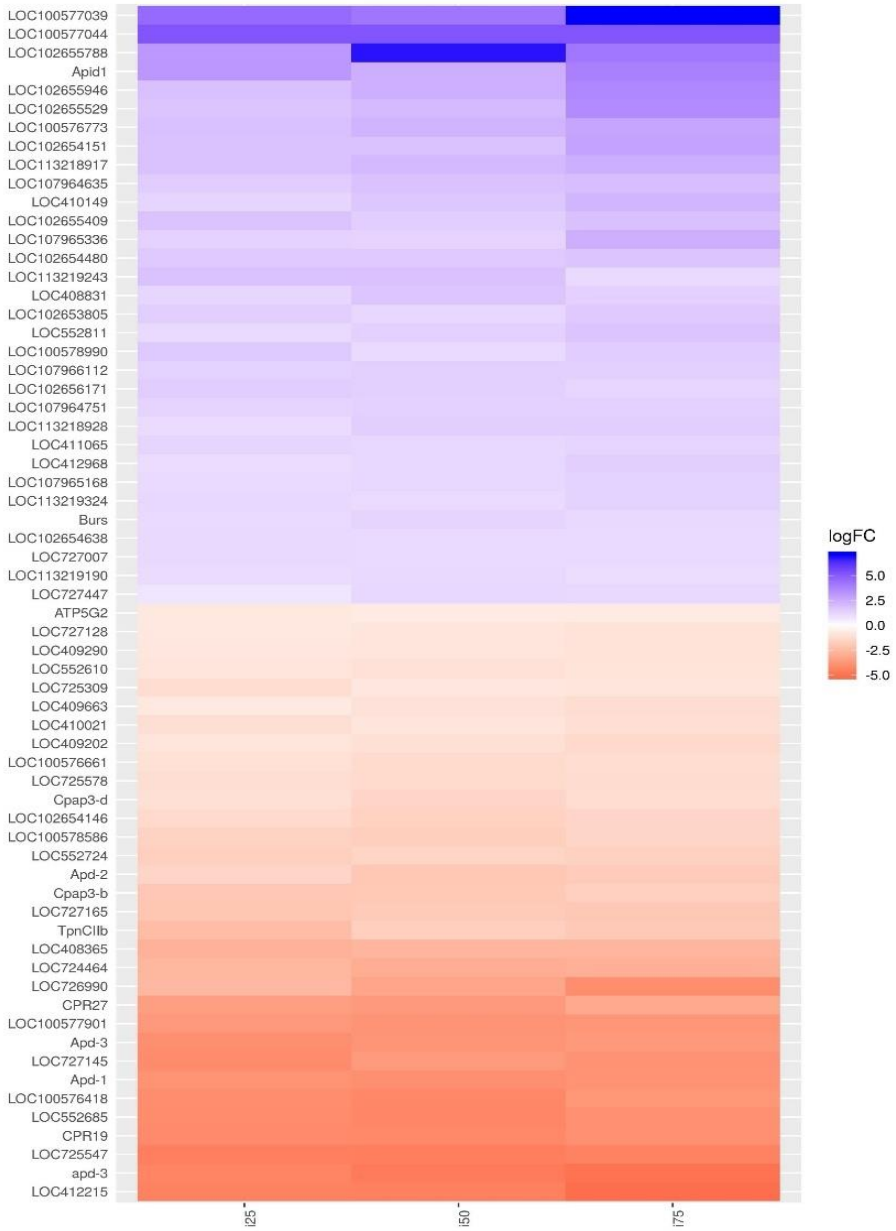

Fig. 7: Clustering analysis of the differentially expressed genes between experimental groups supplemented with inorganic source of zinc and control group.

Regarding the groups supplemented with organic zinc, we observed less enrichment of pathways when compared to treatments that received zinc from an inorganic source. However, some modulated genes that are common in the groups that received supplementation with an inorganic zinc source such as Apd-2 and CPR19, showed that the mineral zinc, regardless of the source, can affect metabolic pathways related to extremely important biological processes in *A. mellifera* honeybees.

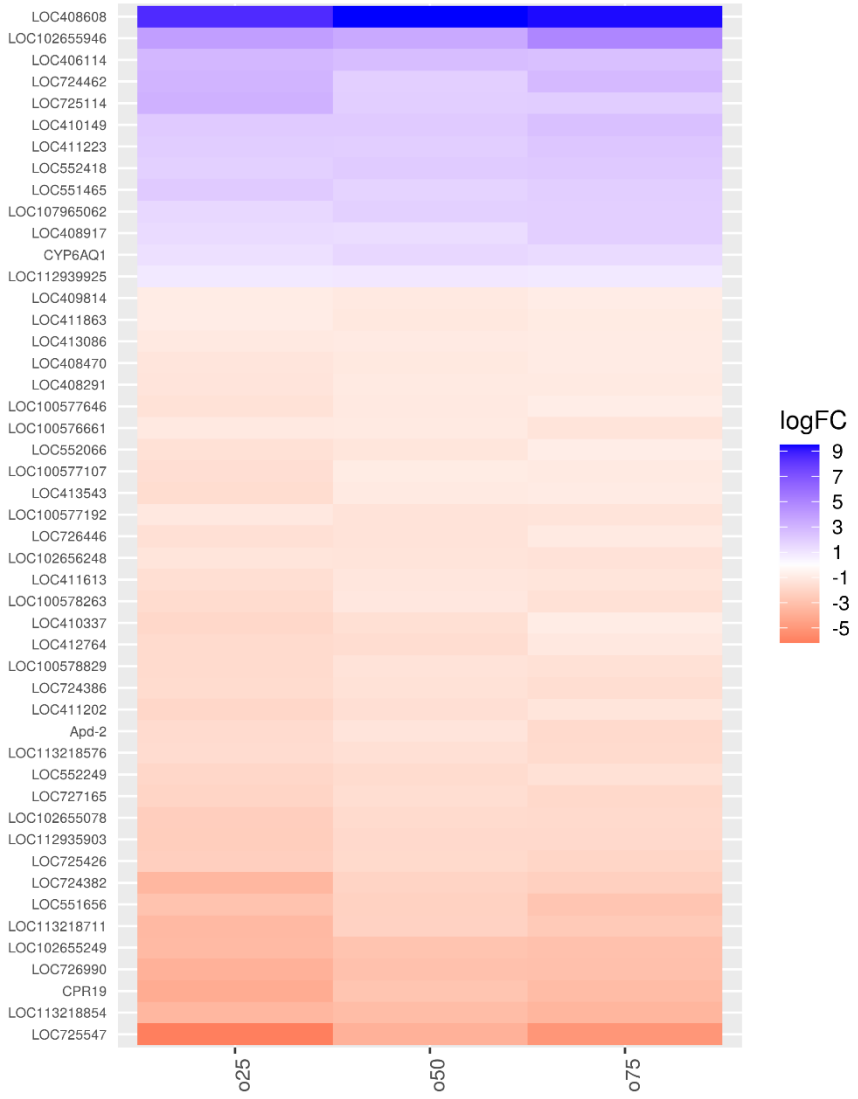

Fig 8: Clustering analysis of the differentially expressed genes between experimental groups supplemented with organic source of zinc and control groups.

Conclusions

Our results indicate that zinc supplementation modulates the expression of many differentially expressed genes with an important role in the development of *Apis mellifera* bees, such as chitin binding and metabolic processes. All the information obtained from this study can contribute to future research in the field of bee nutrigenomics.

Material and Methods

Local

The experiment was conducted at the apiary in the beekeeping 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 area is characterized by a humid subtropical climate with an average altitude of 623 m.

Experimental Groups

Thirty-five beehives of Africanized *A. mellifera* were standardized for the number of breeding and feeding frames and distributed in four treatments (five hives per treatment): n0, control treatment without organic zinc supplementation; ZnO25, supplementation with 25 ppm organic zinc; ZnO50, supplementation with 50 ppm organic zinc; ZnO75, supplementation with 75 ppm organic zinc; ZnI25, supplementation with 25 ppm inorganic zinc; ZnI50, supplementation with 50 ppm inorganic zinc; ZnI75, supplementation with 75 ppm inorganic zinc.

All treatments were supplemented with sugar syrup. However, in the Zn0 treatment, there was no addition of organic zinc, while the other treatments received different concentrations of organic zinc, as described previously.

These values were based on the recommendation of Herbert and Shimanuki [18], who suggested the supplementation of 50 ppm of inorganic zinc.

The organic zinc source used was ferrous zinc methionine (16% zinc) and the inorganic source used was zinc sulfate, which was 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) for one month. The levels provided were confirmed by atomic absorption spectroscopy (FAAS) for the organic and inorganic zinc levels. The following values were obtained: Zn0, 0.00 ppm; ZnO25, 23.16

ppm; ZnO50, 41.07 ppm; ZnO75, 67.44 ppm; ZnI25, 23.5 ppm; ZnI50, 49.4 ppm; ZnI75, 75.09 ppm.

Bee Harvest

At the end of the experimental period, approximately 140 6-day-old bees from 35 hives were collected. For the collection, 12 frames with operculated brood areas were removed, one frame per hive, and then they were wrapped in tissue and placed in an incubator at 30°C and humidity of 60% until the emergence of adults. The emerged workers were marked on the pronotum with a non-toxic pen, while they were attached to the frame. After being marked, the bees were reintroduced into their original hives, and smoke was used to disguise the colony recognition hydrocarbons that could hinder the acceptance of the returning bees. After six days, each bee was collected using an entomological tweezer and immediately stored in plastic pots in an ultra-freezer (−80 °C).

Preparation and sequencing of the RNA-Seq library

Total RNA was extracted from the brain pools of *A. mellifera* nurse bees (20 brains per pool) using the TRIzol® reagent protocol according to the manufacturer's instructions (ThermoFisher Scientific, Waltham, USA). RNA concentrations were quantified in each sample using a QubitTM 2.0 fluorometer (ThermoFisher Scientific, Waltham, USA). The levels of RNA degradation were assessed using a 1% agarose gel. The cDNA libraries were constructed with 200 ng of total RNA using the SureSelect Strand Specific RNA Library Preparation Kit (Agilent Technologies, Santa Clara, USA), following the manufacturer's instructions. The products from the library were sequenced using an Illumina Nextseq platform (Illumina, San Diego, USA) in a single-bore run of 150 bp.

RNA-Seq data processing and difference analysis of genetic expressions

The FASTQC program was used to check the adapter content and assess the quality of raw readings. Data alignment was performed with Burrows-Wheeler Aligner (BWA) v0.7.12, using Amel_HAv3.1 from NCBI (RefSeq assembly accession: GCF_003254395.2) as a reference. The feature count matrix was created using HTSeq v0.11.2 and the GTF annotation file from Amel_HAv3.1. Data analysis, visualization, and plotting were performed using RStudio for the R language, including the ggplot2 v3.3.2 package. Differential expression analysis was performed using the edgeR v3.30.3 package available at Bioconductor software project for the R language. Low expression genes were filtered by keeping genes that had the count per million greater than one in at least two of the samples. The counts were normalized using the TMM normalization. The negative binomial generalized log-linear model was used in the differential expression analysis, and the Benjamini& Hochberg procedure (FDR) was used for multiple testing corrections. Genes with an adjusted p-value < 0.05 were considered to be significantly regulated.

Genetic ontology analysis

Gene ontology analysis was performed for the lists of genes identified with differential expression using the gprofiler2 package, an R interface to the g: Profiler tools (https://biit.cs.ut.ee/gprofiler/gost). Fisher's exact test p-values were adjusted using the Benjamini & Hochberg procedure (FDR) for multiple testing correction, and statistical significance was considered when the adjusted p-value was < 0.05.

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CAPÍTULO 4

Implicações

Há uma grande escassez de informações sobre os minerais na nutrição de abelhas *Apis mellifera*, pouco se sabe sobre os níveis adequados de suplementação, efeitos e fontes minerais que podem ser oferecidas às colônias. Minerais como o zinco são fundamentais como componentes estruturais e funcionais, constituem as metaloproteínas e atuam 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.

O uso de ferramentas moleculares como a metaloproteômica e o transcriptoma podem esclarecer os efeitos de diferentes dosagens e fontes de zinco no funcionamento celular de abelhas *A. mellifera*. Sendo assim, espera-se que este trabalho possa contribuir com informações relevantes na área de nutrigenômica, permitindo a mensuração de níveis adequados de zinco e seus efeitos na dieta de colônias.