

**UNIVERSIDADE ESTADUAL PAULISTA - UNESP
CAMPUS DE JABOTICABAL**

**CARACTERÍSTICAS MORFOMÉTRICAS DE ABELHAS
AFRICANIZADAS: UMA ABORDAGEM QUANTITATIVA E
MOLECULAR**

Marisa Clemente Rodrigues

Mestre em Zootecnia

2020

**UNIVERSIDADE ESTADUAL PAULISTA - UNESP
CAMPUS DE JABOTICABAL**

**CARACTERÍSTICAS MORFOMÉTRICAS DE ABELHAS
AFRICANIZADAS: UMA ABORDAGEM QUANTITATIVA
E MOLECULAR**

Marisa Clemente Rodrigues

Orientador: Prof. Dr. Henrique Nunes de Oliveira

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Campus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Genética e Melhoramento Animal.

2020

R696c Rodrigues, Marisa Clemente
Características morfológicas de abelhas africanizadas: uma abordagem quantitativa e molecular / Marisa Clemente Rodrigues. -- Jaboticabal, 2020
132 p. : il., tabs.
Tese (doutorado) - Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal
Orientador: Henrique Nunes de Oliveira
1. Apicultura. 2. Melhoramento Genético. 3. Marcador Molecular. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp.
Biblioteca da Faculdade de Ciências Agrárias e Veterinárias,
Jaboticabal. Dados fornecidos pelo autor(a).
Essa ficha não pode ser modificada.



UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Jaboticabal



CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: CARACTERÍSTICAS MORFOMÉTRICAS DE ABELHAS AFRICANIZADAS: UMA ABORDAGEM QUANTITATIVA E MOLECULAR

AUTORA: MARISA CLEMENTE RODRIGUES

ORIENTADOR: HENRIQUE NUNES DE OLIVEIRA

Aprovada como parte das exigências para obtenção do Título de Doutora em GENÉTICA E MELHORAMENTO ANIMAL, pela Comissão Examinadora:

Prof. Dr. HENRIQUE NUNES DE OLIVEIRA (Participação Virtual)
Departamento de Zootecnia / FCAV / Unesp - Jaboticabal

Pesquisador Dr. ROBERTO CARVALHEIRO (Participação Virtual)
Departamento de Zootecnia / FCAV / UNESP - Jaboticabal

Prof. Dr. DANISIO PRADO MUNARI (Participação Virtual)
Departamento de Engenharia e Ciências Exatas (DECEX) / FCAV / Unesp - Jaboticabal

Prof. Dr. SAMIR MOURA KADRI (Participação Virtual)
Departamento de Produção Animal / FMVZ/UNESP - Botucatu

Prof. Dr. GREGÓRIO MIGUEL FERREIRA DE CAMARGO (Participação Virtual)
Departamento de Zootecnia / Escola de Medicina Veterinária e Zootecnia da Universidade Federal da Bahia (UFBA) - Salvador/BA

Jaboticabal, 30 de novembro de 2020

DADOS CURRÍCULARES DO AUTOR

MARISA CLEMENTE RODRIGUES – nascida em 17 de dezembro de 1990, na cidade de Tomar – Santarém, Portugal, filha de Maria Ivone Anjos Clemente e Mário Rui Ferreira Rodrigues. Iniciou em setembro de 2009 o curso de graduação em Ciência e Tecnologia Animal na Universidade de Évora em Portugal, obtendo o título de Zootecnista em março de 2013. Em setembro de 2012, ingressou no mestrado em Engenharia Zootécnica na mesma Universidade, obtendo o título de mestre em 2015. Realizou no ano de 2013-2014, um intercambio internacional na Universidade Tecnológica Federal do Paraná, campus de Dois Vizinhos. Ainda na mesma Universidade, ingressou no Programa de Pós-graduação em Produção Animal, focando o seu projeto de pesquisa em melhoramento genético animal de abelhas melíferas. Concluiu o segundo mestrado em agosto de 2016. Em março de 2017, ingressou no curso de doutorado no Programa de Pós-graduação em Genética e Melhoramento Animal na Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista “Júlio de Mesquita Filho”. Recebeu bolsa da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), durante os primeiros três anos do curso.

Kukhala N'ghutsanzaya
"A vida é bonita de viver" na língua Sena

"Ignoramus et ignorabimus"

Emil du Bois-Reymon

DEDICATÓRIA

Dedico e ofereço a minha tese de doutorado à minha estrelinha, avó Júlia.

Maior laço de amor e inspiração da minha vida. Obrigada por tudo!

AGRADECIMENTOS

Agradeço...

Ao determinismo da natureza, pela oportunidade de vivenciar sensações tão incríveis.

Ao meu porto seguro: minha mãe, meu pai, maninho, meus avós, tios e primos, por me darem o apoio, estímulo e perseverança sempre que preciso. À prima Sancho por ser o suporte e incentivo durante esta caminhada.

Ao meu orientador, Prof. Dr. Henrique Nunes de Oliveira, pela confiança, encaminhamento e ajuda em encontrar o foco sempre que precisei e por me ter permitido “abrir asas e voar” em prol do que acredito. Eternamente grata.

Ao Fil, pelos “sabias que” de uma vida e por esta meta que começamos e construímos juntos.

À minha Maria Camila, que é simples e gentil, forte e inteligente, pela inspiração diária e reflexões constantes que me tornam num ser melhor a cada dia.

À Mareliza, a pessoa que me entende no silêncio. A ela devo a amizade e harmonia de partilha diária, enquanto em Jaboticabal.

Aos amigos que acompanharam esta jornada de longe mas que estão sempre lá para me apoiar, Joanica, Carlos, Qui, António, Roberta, Fran, Maira, Jacke, Jéssica, Manu, pelas alegrias e risadas de sempre que fazem as pequenas coisas importantes da vida.

Aos novos amigos que Jaboticabal e o Brasil me deu nesta fase, Mayara, Naty, Lúcio, Cher, Valen, Valter, Telê, Hagabo e Giselly, que me dão forças para sempre continuar. Em especial à família Faria que me acolheu sempre que precisei de me sentir acarinhada.

À minha família angolana, Alicia, mãe Calita, mãe Teresa, mãe Maria, mana Ema, afilhados, Jessika, e todas as minhas “little bees” e apicultores, o mundo é melhor com vocês. À família do Parque Nacional da Gorongosa, o novo projeto que abracei, que me deu apoio e compreensão na última etapa do doutoramento. Ao Matt por me ter permitido sonhar.

À Prof. Dr. Zilá Luz Paulino Simões, por me ter recebido no seu laboratório no início do experimento, pela ajuda, ensinamentos e atenção.

À Prof. Dr. Anete Lourenço, pela paciência e palavras doces todas as vezes que me recebeu no laboratório da USP e por me ter introduzido ao mundo da genética molecular.

Ao Marcus Vinícius pela incansável ajuda com as análises e palavras inspiradoras sempre que a coragem ameaçava ir embora.

Ao Diercles e André que me auxiliaram incansavelmente nas atividades laboratoriais para que este projeto se concretizasse.

A todos os estagiários que ajudaram a fundar o nosso grupo de Estudos e Pesquisas em Abelhas - Flora, pelo convívio e contemplação diária, temos a oportunidade de trabalhar no lugar mais bonito da FCAV, que privilégio!

Aos apicultores e amigos das abelhas que contribuíram com material, abelhas e força motivacional para que pudesse ultrapassar todas as barreiras e realizar o experimento, especialmente ao Cecchetto e sua família maravilhosa.

A todos os alunos que passaram pelas disciplinas que ministrei na UNESP de 2017 a 2019, me fizeram uma melhor profissional.

À Universidade Estadual Paulista, diretor e vice-diretor do campus e Prof. Dr. Alex Maia, pela oportunidade e confiança que depositaram em mim para que pudesse fazer parte da história do setor de apicultura da faculdade.

À CAPES pela bolsa de estudos concedida, espero retribuir ao Brasil a oportunidade que me foi dada de crescimento pessoal e intelectual.

“No final de contas, nós vamos conservar apenas o que amamos; e nós só amamos o que conhecemos; e só conhecemos o que nos é ensinado.” (Baba Dioum). Por isso, agradeço a todos os professores que contribuíram para a minha formação, em especial aos professores que constituíram a banca de avaliação do projeto inicial do doutorado e qualificação. Obrigada pelos exemplos e dedicação. Agradeço também a todos os funcionários da FCAV-Unesp/Jaboticabal pela ajuda e incentivo, principalmente na reativação do setor de apicultura. Vocês fizeram o impossível acontecer.

APOIO FINANCEIRO

À coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela bolsa de doutorado concedida durante o meu percurso acadêmico.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

DESENVOLVIMENTO DO DOUTORADO

Em Setembro de 2016 cheguei a Jaboticabal pela primeira vez, cheguei sem expectativas mas curiosa e confiante naquilo que tinha sido o conselho do Prof. Gregório Camargo, que me inspirou a seguir a vida acadêmica em vez de voltar para a Nova Zelândia, onde eu trabalhava na indústria da criação de rainhas. De papel na mão, onde eu tinha uns rabiscos com ideias daquilo que gostaria que fosse o meu projeto de doutorado, me apresentei ao professor Henrique Nunes de Oliveira. Foi a partir daí que tudo começou.

Desde o projeto proposto para ingressar no doutorado, a minha ideia era continuar com estudos em genética e melhoramento de abelhas mas desejava entrar no mundo da genética molecular para ganhar experiência e tentar resolver algumas questões que me intrigaram ao longo do meu percurso acadêmico. Para além disso, não queria deixar de lado aquilo que tinha sido a minha experiência profissional em relação à extensão e desenvolvimento rural que comecei a desenvolver logo após o final da minha graduação.

Durante o mestrado, na Universidade Tecnológica Federal no Paraná, aprendi a conduzir experimentos, realizar análises e coletas, lidando com as dificuldades diárias e me adaptando a um novo lugar. Durante o mestrado trabalhei em um projeto para estimação de parâmetros genéticos para zangões, que normalmente são a casta menos estudada das abelhas *Apis mellifera*. No laboratório onde eu estudava, várias questões tinham sido levantadas sobre rainhas e critérios de seleção para usar em programas de melhoramento. Isso levou-me a pensar naquilo que seria, mais tarde, o meu projeto de doutorado. Baseada na hipótese: Se rainhas com valor genético superior para peso, possivelmente, darão origem a colônias com maior produção de mel, então, como é que a genética molecular nos pode ajudar a desvendar um pouco mais a pontinha desse “iceberg”? Foi com esta exata questão, e depois de muita leitura, que as ideias surgiram. Meus maiores desafios eram: aprender sobre as metodologias moleculares, aplicá-las e poder comparar os resultados. Assim, seria capaz de responder à pergunta que me motivara a seguir o percurso acadêmico.

Os maiores desafios estavam prestes a começar, era preciso material para trabalhar, era preciso um setor de apicultura funcional e uma equipe. E

no momento, essa estrutura não existia na Faculdade de Ciências Agrárias e Veterinárias (FCAV). Inspirada pela história do setor de apicultura da FCAV, nos últimos 35 anos, e com o apoio e confiança do professor Henrique, meu orientador, e do professor Alex, chefe do Departamento de Zootecnia, naquela data, me senti confiante de “embarcar” na aventura, não só da pesquisa mas de reunir esforços para criar um espaço que viria, uns meses depois, a ser a casa do nosso Grupo de Estudos e Pesquisas em Abelhas – Flora, da FCAV-UNESP. Desde o arranque do setor até ao final do meu doutorado, mais de 20 alunos da graduação e pós-graduação passaram pelo laboratório para estagiar e/ou realizar treinamentos ou conduzir projetos de pesquisa.

Para finalizar, acho importante manifestar a minha felicidade e gratidão à estrutura da UNESP e a todos os professores pela oportunidade que tive em vivenciar, em 3 anos, o que considero ser o triângulo base da academia: Pesquisa, Ensino e Extensão:

Na **Pesquisa**, tive a oportunidade de executar não só o meu projeto de doutorado mas também de colaborar com vários projetos de TCC e estágios curriculares dos alunos da graduação e mestrado. Juntamente com os alunos, fundamos o que é hoje o Grupo de Estudos e Pesquisas em Abelhas, a que chamamos Flora.

Realizei parceria com a empresa Tijolo Ecoterm, de onde surgiu a ECOLMEIA, fabricada com tijolo ecológico. Conseguimos construir 12 colmeias que estão hoje sendo usadas para experimentos no setor.

A convite do Departamento de Entomologia Agrícola, escrevi um capítulo de livro intitulado “Importância dos Polinizadores no Desenvolvimento Rural”, publicado em 2019, no livro: XI Tópicos em Entomologia Agrícola.

Tive também a oportunidade de autoria em um capítulo: “The Origin of European Bees and their Intraspecific Biodiversity” no livro *Phylogenetics of Bees*, publicado em 2019 pela editora CRC Press Taylor & Francis Group.

No **Ensino**, ministrei duas disciplinas, Apicultura e Sericicultura, para a graduação em Zootecnia, de 2017 a 2019, fazendo um total de 270 horas de aula ministrada.

Na **Extensão**, tive oportunidade de ministrar um curso de treinamento para apicultores rurais do projeto “From Bee-burners to Beekeepers”, da ONG

Fauna and Flora International, na ilha do Príncipe, em São Tomé e Príncipe, na África, em dezembro de 2017.

Consegui também dar continuidade ao projeto de produção de mel sustentável em Angola no qual participo desde 2016, ainda que mais à distância do que presencialmente. Desse projeto, surgiu o trabalho em parceria com o Ministério do Ambiente de Angola e com o projeto internacional Save the Elephants: <http://elephantsandbees.com/bee-hive-fence/angola/>, para ajudarmos a mitigar os conflitos Homem-Elefante, através da utilização de colmeias suspensas. Consegui um Small-Grant Award (da organização Save the Elephants) para construirmos 4 cercas de colmeias suspensas, atualmente implementadas no norte de Angola. Ainda em Angola, no ano de 2020, participei na Expedição do Projecto Okavango Wilderness da National Geographic, para treinamento dos apicultores locais. Esse projeto culminou na escrita e publicação de um Guia Ilustrado para crianças, intitulado: “Guia de Coexistência Homem-Elefante-Abelha”.

A UNESP, especialmente o meu orientador e o programa da Pós-Graduação, foram fundamentais para que eu continue a acreditar que é possível trabalhar na pesquisa e na extensão, no fim, uma leva à outra e ambas se enriquecem e complementam. Desse trabalho de extensão surgiu um artigo científico publicado na revista *Journal of Apicultural Research* em 2019: “First detailed report of infestation of African honey bees (*Apis mellifera scutellata*) in Angola by the bee lice *Braula coeca* (Diptera: Braulidae)”.

No Brasil, tive oportunidade de ser uma das mentoras de um projeto de Educação Ambiental que levou as abelhas até à escola Municipal Palma Travassos em Jaboticabal, projeto organizado pela professora Tatiana Souza, coordenadora do Grupo de Estudos em Educação Ambiental – GEEA.

Na fase final do doutorado (2020), foi-me concedida uma bolsa do USDA em Maryland nos USA, para realizar um estágio no *Bee Research Lab*, coordenado pelo pesquisador Dr Jay Evans. Devido à pandemia que atravessamos, a minha ida foi cancelada e acabou por não se concretizar este estágio.

Desde Junho de 2020, atuo como Gestora do Projeto de Produção de Mel e Pesquisa focada em apicultura no Parque Nacional da Gorongosa em

Moçambique, onde tenho oportunidade de culminar tudo o que aprendi a nível acadêmico com a importante componente de extensão rural e ensino.

ESTRUTURA DA TESE

Durante o desenvolvimento do meu doutorado pretendi ter uma abordagem holística para ampliar o meu aprendizado e experiência profissional. Nesse contexto, conduzi o doutorado para que envolvesse não só a componente molecular mas que também fosse possível aprofundar conhecimento sobre o gene em estudo, metodologia laboratorial para histologia dos órgãos reprodutivos de rainhas *Apis mellifera*, análise estrutural do gene e suas possíveis aplicações para avaliação da subespécie em estudo. De acordo com essa abordagem a estrutura final da tese culminou em seis capítulos, sendo o primeiro e o último a introdução e conclusão geral, respectivamente.

No primeiro, apresento as considerações iniciais numa perspectiva abrangente sobre a apicultura nacional, a relevância do melhoramento genético no setor apícola e a complexidade do sistema de acasalamento da espécie em foco.

No segundo, apresento uma revisão bibliográfica, onde fiz o levantamento e a compilação de informações relevantes publicadas até ao momento, sobre a vitelogenina e o seu gene correspondente, *Vg*. Nesse capítulo abordo principalmente a importância do gene no contexto da reprodução em *Apis mellifera* e em outras espécies no qual já foi aprofundadamente estudado em relação à fisiologia da reprodução. Este artigo foi redigido como *Review Article* para ser submetido na revista *Apidologie*.

No terceiro capítulo apresento um protocolo desenvolvido durante o doutorado para contagem de número de ovariolos, presentes nos ovários de rainhas virgens. Este protocolo, prático e acessível, facilitará a abordagem morfométrica em associação com futuros projetos que envolvam seleção genética e/ou avaliação morfológica das rainhas recém-emergidas. Este artigo foi redigido como *Short-Communication* para ser submetido na revista *Journal of Apicultural Research*.

O quarto capítulo, considero como o culminar do meu projeto de pesquisa, onde apresento os resultados do sequenciamento do gene *Vg* dos indivíduos selecionados pelo valor genético do peso à emergência. Os resultados neste capítulo contribuem para a definição do gene como possível

candidato em associação com características morfométricas. Este artigo foi submetido como *Original Article* na revista *Apidologie*.

No quinto capítulo, recorrendo às amostras dos animais sequenciados, estudei o gene do ponto de vista da sua utilização como passaporte genético para identificar a ancestralidade evolutiva, dos animais amostrados neste trabalho. Este capítulo contribuiu para a redação de um capítulo intitulado “The Origin of European Bees and their Intraspecific Biodiversity” no livro *Phylogenetics of Bees*, publicado em 2019 pela editora CRC Press Taylor & Francis Group.

O sexto capítulo corresponde às considerações finais que resultaram da abordagem conjunta dos capítulos anteriores.

SUMÁRIO

CONSIDERAÇÕES GERAIS	20
1. RESUMO	20
2. ABSTRACT	21
3. INTRODUÇÃO	22
3.1 Panorama atual e estratégias para o desenvolvimento da apicultura brasileira	23
3.2 Melhoramento genético de abelhas <i>Apis mellifera</i>	24
3.3 Particularidades da <i>Apis mellifera</i>	26
3.4 Vitelogenina e o seu papel na colônia	28
4. Conclusão	30
5. Objetivos	31
6. Revisão de Literatura	31
CHAPTER II - Why does vitellogenin matter? A review towards behavior and reproductive biology of honeybees and other insects	40
Abstract	40
Introduction	40
Characterization of Vg	41
How it acts	43
Vitellogenin and its key role in reproduction and life cycle	45
Gene structure	49
Vitellogenin gene knockdown	53
Vitellogenin and its role in longevity and fertility	54
Role on reproduction and behavior: other orders, other species	56
Coleoptera	57
Hemiptera	58
Hymenoptera	60
Diptera	62
Lepidoptera	63
Prospects: Epigenetics affecting the expression of Vg	65
Conclusion	66
References	67
CHAPTER III - A rapid and inexpensive histological procedure for reproduction associated-trait in <i>Apis mellifera</i> virgin queens	75
Histological Procedures	76
Fixation, Dehydration, Diaphanization and Infiltration	76
Paraffin removal, Rehydration, Staining and Dehydration	78

References	80
CHAPTER IV - Association between <i>Vg</i> gene polymorphisms and weight at emergence in <i>Apis mellifera</i> queens	83
Abstract	83
Introduction	84
Material and Methods	85
Animals	85
Genotyping and sequencing	86
Estimates of genetic parameters for weight at emergence	87
Verification of SNPs	88
Results	89
Discussion and Conclusion	91
References	92
Tables and Figures	96
Supplementary material	100
CHAPTER V – Genetic passport of honey bees towards vitellogenin (<i>Vg</i>) SNP markers	106
Abstract	106
References	114
Supplementary Material	117
CAPÍTULO VI - Considerações Finais	131

CAPÍTULO I

Considerações Gerais

CONSIDERAÇÕES GERAIS

1. RESUMO

Nas abelhas *Apis mellifera* L., a performance reprodutiva das rainhas é representada por um conjunto de características com cada vez maior relevância na apicultura mundial devido ao alto valor econômico associado. Os parâmetros genéticos para as características associadas à reprodução são muito importantes pois apresentam, na abelha africanizada, interessantes correlações genéticas com algumas características morfométricas, como é o caso do peso à emergência com o peso dos ovários, o que pode permitir o ganho genético por seleção. Contudo, não existem muitos trabalhos que aliem a estimação de parâmetros genéticos com análises genômicas de forma que os aspectos fisiológicos sejam compreendidos ao nível quantitativo e molecular, simultaneamente. Nesse trabalho propõe-se fazer a estimação de parâmetros genéticos para o peso da rainha à emergência e identificação de polimorfismos no gene previamente indicado como candidato: gene da vitelogenina (*Vg*), para as características supramencionadas em rainhas de abelhas africanizadas. Dessa maneira, contribuir-se-á para um melhor entendimento sobre o gene e para a avaliação genética de rainhas com a compreensão dos processos fisiológicos que influenciam a sua performance reprodutiva, a fim de melhorar o desempenho da apicultura nacional e alavancar novas pesquisas sobre a biologia reprodutiva das abelhas.

Palavras-chave: *Apis mellifera*, reprodução, SNP, vitelogenina.

2. ABSTRACT

In *Apis mellifera* L. bees, the reproductive performance of queens is characterized by a set of traits with increasing relevance in international beekeeping due to its high associated economic value. Genetic parameters for reproduction-associated traits are very important, since present, in the Africanized honeybees, interesting genetic correlations with some morphometric traits, such as emergence weight with the weight of the ovaries. However, there are not many studies that include estimation of genetic parameters with genomic approaches so that the physiological aspects are understood at the level of quantitative and molecular genetics, simultaneously. In this present study, I proposed to estimate genetic parameters for the queen's weight at emergence and also identify polymorphisms in the gene previously indicated as a candidate: vitellogenin gene (*Vg*), for weight at emergence. In this way, we aim to contribute to a better understanding of the gene approaching physiological processes that influence their reproductive performance, in order to improve national beekeeping and leverage and inspire new research focused to unravel the reproductive biology of bees.

Keywords: *Apis mellifera*, reproduction, SNP, vitellogenin.

3. INTRODUÇÃO

As abelhas da espécie *Apis mellifera* L. são das mais importantes para a manutenção da produtividade de culturas vegetais de interesse econômico por meio da polinização (Dos Santos et al. 2018). No entanto, na última década, a apicultura tem sido um aspecto mediático devido ao atual declínio que se tem vindo a registrar no número de colônias no mundo ocidental, inclusive no Brasil (Lu, et al. 2020). Por isso, torna-se evidente a necessidade de compreender de que forma se podem desenvolver metodologias que promovam a apicultura mundial. Cobey et al. (2012) defendem que algumas soluções passam pelo delineamento eficaz de programas de melhoramento genético. Estes programas devem considerar em seus objetivos a obtenção de colônias com maior resistência a doenças e parasitas, utilizando, dessa forma, uma abordagem genética para assegurar uma população controlada e sustentável a longo prazo.

Contudo, a indústria apícola tem pouco acesso a programas de melhoramento genético padronizados e, como consequência, não se beneficia de forma igual dos resultados que estes programas podem propiciar como acontece nas indústrias de outras espécies pecuárias. No entanto, as pesquisas científicas em melhoramento genético de *Apis mellifera* têm sido dependentes do financiamento institucional e direcionadas para o estudo e seleção de características indicadoras de produção, as quais são, normalmente, difíceis e morosas de mensurar (Cobey et al. 2012). A investigação tem neste setor um papel fundamental pois funciona como veículo para resolver questões como a produção de rainhas, produção de zangões, estabelecimento de linhagens, entendimento dos processos fisiológicos e anatômicos, contribuindo para a profissionalização da atividade.

3.1 Panorama atual e estratégias para o desenvolvimento da apicultura brasileira

A apicultura no Brasil tem apresentado progressivo crescimento, explicado pela introdução de abelhas africanas (*Apis mellifera scutellata* L.) em território brasileiro, na década de 50 (Kerr, 1957; 1967). A partir de então foi iniciado um fenômeno de cruzamentos naturais com as abelhas de origem europeia (*A. m. mellifera*, *A. m. ligustica*, *A. m. carnica* e *A. m. caucasica*), propiciando a formação de um poli-híbrido, atualmente chamado de abelha africanizada (Soares, 2012). Como resultado tem-se grande variabilidade genética, com predominância das características morfológicas das abelhas europeias no sul do país, enquanto no norte predominam as características morfológicas e comportamentais das abelhas africanas (Pereira et al. 2003).

O Brasil detém imenso potencial apícola, pela sua extensão territorial, floradas diversificadas e condições climáticas, possibilitando o manejo durante todo o ano e fácil adaptação das abelhas africanizadas às condições tropicais (Nogueira-Couto e Couto, 2006). Adicionalmente, o setor apícola tem desenvolvido estímulos de organização e aprimoramento técnico, envolvendo pesquisadores, apicultores, órgãos governamentais e não governamentais (Costa-Maia e Lino, 2009). Desse modo, a apicultura apresenta-se como um setor chave para o fortalecimento da cadeia produtiva agrícola relacionada ao desenvolvimento sustentável do setor ambiental, econômico e social do país.

Entre os diversos produtos da produção apícola destaca-se o mel, um produto alimentício que agrega de maneira quantitativa e econômica a cadeia (Silveira-Júnior et al., 2020). Comercialmente, o mel ocupa lugar de destaque no mercado nacional e internacional e pelo seu elevado padrão de qualidade, o mel brasileiro tornou-se um produto muito apreciado pelos principais mercados internacionais apícola (de Figueiredo et al., 2016). Segundo a FAO (2018), o Brasil foi o 11º maior produtor mundial e 9º maior exportador, sendo que no ano de 2018, foram registradas cerca de 42,3 mil toneladas de mel produzidas e 28 mil toneladas exportadas. Gerando uma receita de ~ R\$ 502,8 milhões, apresentando um valor médio pago pelo quilo do mel exportado de US\$ 3,34 (IBGE, 2019 e Comex 2019). Frente a esses avanços e tendências

do mercado externo, há motivações por parte dos apicultores para o aumento de suas produções. Desta forma, a procura por novas abordagens tecnológicas tem priorizado o futuro sustentável das populações de *Apis mellifera* a médio/longo prazo. E, visando a obtenção de linhagens que apresentem valor genético superior para características desejáveis, a planificação de programas de melhoramento genético tem recebido destaque mundial (Cobey et al. 2012).

3.2 Melhoramento genético de abelhas *Apis mellifera*

O principal problema metodológico associado ao melhoramento da *Apis mellifera* está relacionado com o desempenho e comportamento da colônia, uma vez que estes resultam da interação entre rainha e operárias (Bienefeld et al. 2007). Segundo Fuchs e Schade (1994) os programas de produção apícola têm de ser baseados na seleção de características comportamentais ao nível da colônia. Portanto, devem ter-se em consideração a dinâmica social dessa, o acasalamento das rainhas, as consequências da endogamia e as influências ambientais.

De acordo com Page e Laidlaw (1997) as questões decisivas para obter sucesso no melhoramento genético em abelhas por meio da seleção são (i) a escolha das colônias – devem ser identificadas e devem existir diferenças que promovam o potencial da população parental, (ii) a manutenção da variabilidade genética, (iii) o controle de acasalamentos, (iv) o rigor na manutenção das genealogias durante todo o programa, ao que Costa-Maia et al. (2011) adicionaram (v) a estimação precisa de parâmetros genéticos para a predição de valor genético individual e posterior identificação dos animais geneticamente superiores.

As estimativas dos parâmetros genéticos podem variar consideravelmente como consequência das diferenças genéticas da população, do ambiente, do tipo de análise e do método de estimação de componentes de (co) variância. Há portanto, necessidade de avaliar esses parâmetros separadamente na população em estudo.

Chevalet e Cournet (1982) adaptaram a *A. mellifera* o modelo desenvolvido por Willham (1963), que separou as estimativas de herdabilidades para efeito direto e materno. Bienefeld e Pirchner (1990)

estimaram parâmetros genéticos por meio do método de quadrados mínimos e encontraram herdabilidades para os efeitos operária e rainha na produção de mel (0.26 e 0.15), cera (0.39 e 0.45), para variáveis associadas ao comportamento defensivo (0.41 e 0.40) e desenvolvimento colonial primaveril (0.76 e 0.46), respectivamente. Bienefeld e Pirchner (1990) derivaram o índice de seleção para várias características considerando simultaneamente os efeitos de rainha e operárias. Bienefeld et al. (2007) consideraram que o uso de índices de seleção não estava adequado em função das influências ambientais e diferenças genéticas nos níveis de acasalamentos. Nesse seguimento, os autores, sugeriram a utilização do Modelo Animal 'BLUP' (*Best Linear Unbiased Prediction*) com adaptações pertinentes às abelhas.

Hoopingarner e Farrar (1959) encontraram uma forte correlação fenotípica entre o peso da rainha e número de ovariolos. Portanto, a partir deste momento o peso à emergência começou a ser indicado como característica possivelmente relacionada à performance reprodutiva. Le Conte (2001), constatou que rainhas mais pesadas possuem maior capacidade de oviposição, esse trabalho foi corroborado por Winston (2003) pela afirmação de que o peso da rainha está relacionado ao desenvolvimento das estruturas reprodutivas, como os ovários e a espermateca, podendo o peso, ser por isso, um dos indicadores da qualidade da performance reprodutiva das rainhas, de um ponto de vista fenotípico. Fraquinello (2007), por meio de estimativas de parâmetros genéticos, concluiu que existe potencial de seleção para aumentar a produção de geleia real usando como critério a largura do abdômen das rainhas recém-emergidas.

Rodrigues (2016) fez um levantamento das estimativas de herdabilidades publicadas, a partir de 1978, para a produção de mel em *Apis mellifera* L. estas variaram entre 0.16 a 0.75 (Pirchner et al. 1960; Pirchner e Ruttner, 1962; Vesely e Siler, 1964; El-Banby, 1967; Soller e Bar-Cohen, 1967; Börger, 1969; Zawilski, 1974; Malkov e Sedykh, 1980; Collins et al. 1984; Oldroyd et al. 1987; Bienefeld e Pirchner, 1990; Willam e EBI, 1993; Mostajeran et al. 2000; Costa-Maia, 2009; Padilha, 2013). Utilizando o modelo animal, Costa (2005) avaliou parâmetros genéticos e fenotípicos em rainhas africanizadas para o peso à emergência e os comprimentos e larguras da asa e abdômen e verificou potencial de seleção fenotípica em todas as

características.

Costa-Maia (2009) estimou parâmetros genéticos para a produção de mel, peso e características morfométricas da rainha, considerando o efeito genético materno. As herdabilidades encontradas para o peso da rainha à emergência e para a produção de mel foram 0.54 e 0.34, respectivamente. Num estudo sobre aspectos genéticos de características morfométricas e reprodutivas de rainhas, Martins (2014) concluiu que a seleção pelo peso à emergência deve resultar em rainhas com ovários mais pesados, indicando que essa característica pode ser utilizada em programas de melhoramento genético visando maior potencial reprodutivo de rainhas de *A. mellifera*.

Brascamp e Bijma (2014) mostraram como a matriz de parentesco e a sua inversa podem ser usadas para estimar valores genéticos e componentes de variância para populações de abelhas. Costa-Maia et al. (2015) recorrendo à abordagem Bayesiana, encontraram estimativas de herdabilidade de 0.70, 0.68, 0.69, 0.63, 0.67, 0.68 e 0.70 para o peso à emergência, o peso à maturidade, a área, volume e peso das glândulas do muco e a área e volume das vesículas seminais de zangões, respectivamente. Por meio das correlações genéticas estimadas pelos autores, como o peso à emergência e o volume das vesículas seminais (0.44), concluiu-se que a seleção de zangões com valor genético superior para peso à emergência pode proporcionar zangões com maior capacidade de armazenamento de sêmen.

3.3 Particularidades da *Apis mellifera*

A variabilidade genética dentro de uma população é um reflexo direto do número de zangões que acasalam com cada rainha (Tarpay et al. 2000) e é importante, pois maior variabilidade resulta em maior potencial de resposta à seleção e num progresso genético mais rápido (Rinderer e Collins, 1986). O melhoramento genético visa o aumento das frequências alélicas de alelos favoráveis dos *loci* de importância econômica a serem selecionados na população em estudo (Rinderer, 1977; Page e Laidlaw, 1982; Moritz, 1986; Bienefeld et al. 2007). No entanto, a avaliação genética em abelhas tem sido pouco realizada, ao contrário do que tem acontecido em outras espécies de animais, sobretudo devido às peculiaridades do seu sistema de acasalamento (Costa-Maia et al. 2011; Cobey et al. 2012; Brascamp e Bijma, 2014).

Nas colônias de *A. mellifera* L. geralmente existem rainhas, operárias e zangões. Cada tentativa de iniciar um novo ciclo reprodutivo começa com uma rainha virgem, sendo que alguns dias após a sua emergência, esta deixará temporariamente a colônia para realizar um (ou mais) voo(s) nupcial(ais) em que acasalará com vários machos reunidos em zonas de congregação. Os machos morrem imediatamente após o acasalamento, acasalando apenas com uma rainha. Após a fecundação a rainha armazena na sua espermoteca os espermatozóides que utilizará durante toda a sua vida produtiva (Moritz e Southwick, 1992). Durante esse período, poderá originar dois tipos de ovos: fertilizados e não fertilizados.

As rainhas e as operárias são indivíduos diploides, sendo que os zangões, que sobrevivem naturalmente na colônia são haploides, a *A. mellifera* adota assim a haplodiploidia (presente na Ordem Himenoptera a que pertence) como sistema de determinação sexual. Dado que a descendência masculina herda apenas material genético proveniente da sua mãe (Laidlaw e Page, 1984), os machos podem ser considerados gametas da progenitora (Brascamp e Bijma, 2014). Considerando o hábito de acasalamento múltiplo da rainha e a natural existência de machos haploides, em uma colônia o parentesco dos indivíduos, nela originados, pode variar entre 0.25 e 0.75 (Crow e Roberts, 1950; Polhemus et al. 1950; Laidlaw e Page, 1984; Moritz e Southwick, 1992; Bienefeld et al. 2007).

O coeficiente da relação de parentesco (probabilidade de que em um *locus* escolhido ao acaso, o indivíduo 'B' partilhe um alelo idêntico ao do indivíduo 'A') entre rainha 'A' e zangão filho 'B' (0,5) é diferente do coeficiente de relação de parentesco entre zangão 'A' e rainha mãe 'B' (1), que indica a assimetria no relacionamento entre a rainha e a sua descendência masculina. Isto significa que apenas metade dos genes da rainha estão representados em cada um dos seus filhos zangões, mas todos os genes de cada um dos seus filhos foram dela herdados (Moritz e Southwick, 1992). O coeficiente de coancestralidade é a relação de parentesco entre um indivíduo e os próprios gametas, que entre rainha e zangão é igual a 0,5, mas o cálculo da relação de parentesco, que inclui o coeficiente de parentesco dos zangões, é igual a $\frac{1}{\sqrt{2}}$, aproximadamente 0,707 (Moritz e Southwick 1992). Como os zangões são

haploides e por isso os seus espermatozoides são cópias do seu genótipo, os termos da amostragem Mendeliana entre irmãos estão correlacionados e a matriz de covariância da amostragem Mendeliana não é diagonal (Brascamp e Bijma 2014).

A diferenciação das castas femininas é um dos assuntos mais estudados em relação aos processos reprodutivos em *Apis mellifera*. Especialmente as redes integrativas de microRNA, têm sido relatadas por terem efeito no desenvolvimento dos ovários e com isso causar diferenças fenotípicas entre rainha e operárias (Ashby et al. 2016). No estudo de Xiao e Wei (2020). Para se tornar uma rainha, uma larva deve ser alimentada com uma dieta especial, em uma maior quantidade e por um período de tempo mais longo do que larvas que vão originar operárias. Essas diferenças na dieta provocam alterações endócrinas que induzem um processo de desenvolvimento específico da casta (Ruden et al. 2015). Na abelha melífera, essa sequência de eventos foi estudada em detalhe em associação com o desenvolvimento dos ovários.

Um elevado nível de hormônio juvenil durante a maior parte do período larval garante a sobrevivência dos ovários durante a metamorfose até ao imago, dando origem a rainhas com ovários completos com 100 a 180 ovariolos por ovário (Rachinsky et al. 1990; Jackson et al. 2011). Em contraste, nas operárias apenas alguns ovariolos (2-12) sobrevivem ao processo da metamorfose (Hartfelder e Steinbruck, 1997; Schmidt-Capella e Hartfelder, 1998). Outro aspeto importante na diferenciação da casta feminina está relacionado com a vitelogenina, a principal proteína envolvida na formação de ovos, presente em zangões, operárias e rainhas (Mutti et al. 2011).

A síntese de vitelogenina tem início mais cedo no desenvolvimento das rainhas, começando 60 horas antes da sua emergência e 10 horas antes nas operárias (Piulachs et al. 2003). De acordo com os autores, pouco depois da emergência, a vitelogenina começa a acumular-se e em três dias representa até 60% das proteínas da hemolinfa, proporção que se mantém durante toda a vida adulta da rainha.

3.4 Vitelogenina e o seu papel na colônia

A vitelogenina é uma proteína precursora do vitelo, presente no ovo, similar às lipoproteínas nos vertebrados (Baker, 1988; Mann et al. 1999). Nos

insetos, ela é sintetizada principalmente no corpo gorduroso e libertada na hemolinfa, de onde é sequestrada pelos oócitos em desenvolvimento (Piulachs et al. 2003). A síntese de vitelogenina está intimamente relacionada à maturidade sexual das fêmeas, embora machos e fêmeas parcialmente estéreis, também sejam capazes de sintetizá-la. O gene da vitelogenina (*Vg*) da abelha codifica a proteína de mesmo nome, que afeta para além da função reprodutiva, o comportamento, a imunidade, a longevidade, e a organização social das colônias de *Apis mellifera* (Munch e Amdam, 2010; Nunes et al. 2013). Os efeitos pleiotrópicos específicos da vitelogenina na fisiologia das abelhas sugerem que esta proteína pode suprimir a sinalização de insulina nas operárias e condicionar o forrageamento (Seehuus et al. 2006; Hunt et al. 2007; Ament et al. 2008; Wang et al. 2010). Faz-se interessante proceder a investigação de alterações no gene da vitelogenina dado a sua ação fisiológica multidisciplinar.

Em um estudo relativamente recente, Ilyasov et al. (2015) mostraram a existência de 26 SNPs (*Single Nucleotide Polymorphism*) no gene *Vg*, mostrando diversidade genética entre subespécies de *Apis mellifera*. Todavia, a possível influência destes marcadores moleculares na expressão de proteínas, não foi observada no estudo. Os autores ainda relataram a contribuição dos polimorfismos na diferenciação de ramos evolutivos no estudo de algumas linhagens de *Apis mellifera*, enfatizando ainda mais, a importância do seu estudo. Compreender o efeito desses SNPs, na diferenciação entre linhagens evolutivas da espécie, pode ajudar no entendimento de genética populacional e, conseqüentemente, auxiliar em programas de melhoramento genético em *A. mellifera* (Qin et al. 2006). Delaney et al. (2011) constataram que os níveis de transcrição de vitelogenina podem estar associados ao peso da rainha. Tanaka e Hartfelder (2004) concluíram que esta glicolipoproteína é um dos indicadores do potencial de fecundação, uma vez que é o precursor da gema e pode estar associado à produção de ovos. Em contraste com os genomas de outros insetos sociais, o da abelha contém apenas uma cópia de *Vg* (Kent et al. 2011). O gene *Vg* é constituído por sete exons, as suas sequências estão publicadas no banco de dados GenBank (ver: <http://www.ncbi.nlm.nih.gov>), os indicadores para sua deteção, caracterização e quantificação estão representados na Tabela 1.

Tabela 1. PCR *primers* dos exons do gene *Vg* de abelhas *Apis mellifera* (Kent et al. 2011).

Exon	Primer	
	F 5'-3'	R 5'-3'
2	tctgttcgtccaggtcc	gacagttcagccgactcc
3	ccttcgatccattcctga	gtcaaaacggattggtgctt
4	tcgaaggggaagaatttaa	acgagcaattcctcaacacc
5	gtcggacaatttcacgtcct	gttcgagcatcgacacttca
6	agagccagggatacgtcaaa	gagtcattctcgaggctcacc
7	ttctggctgaggtcaggatt	aatttcgaccacgactcgac

4. Conclusão

De acordo com a literatura, para abelhas africanizadas, (Costa-Maia, 2009; Costa-Maia et al. 2011; Martins, 2014) espera-se que a seleção para a produção de mel baseada no peso da rainha à emergência seja viável, visto que as estimativas de correlação genética entre estas características são de magnitude moderada e positiva (Costa-Maia, 2009). Contudo, se o critério de seleção for a própria produção de mel o progresso genético poderá ser lento, devido à necessidade de aguardar e monitorizar a produção ao longo do ano, havendo conseqüentemente um aumento do intervalo entre as gerações. Assim, pode ser utilizado o peso da rainha à emergência como critério de seleção, com a vantagem da drástica redução do intervalo de geração, trazendo rápido progresso genético à produção de mel por resposta correlacionada. A hipótese associada a esta relação entre o peso da rainha à emergência e a produção de mel, parte do princípio de que rainhas com maior valor genético para peso tenham vantagens reprodutivas, nomeadamente o peso dos ovários e número de ovariolos. O entendimento dos mecanismos fisiológicos por meio das análises genéticas contribuirá fortemente para a

planificação e execução de programas de melhoramento que permitam a melhoria da performance reprodutiva das rainhas e o consequente aumento da produção de mel, obtendo simultaneamente importantes dados para consolidar a informação disponível no panorama genético nacional e internacional da apicultura.

5. Objetivos

Neste estudo os principais objetivos foram:

(i) compreender a importância da vitelogenina e do seu gene correspondente, *Vg*, na biologia reprodutiva de abelhas e outros insetos;

(ii) identificação de SNPs no gene da vitelogenina (*Vg*) para verificar a associação desses com os fenótipos medidos;

(iii) desenvolver ou adaptar um protocolo histológico para contagem de ovariolos em rainhas virgens;

(iv) identificar a ancestralidade/proximidade genética das abelhas sequenciadas por meio do contraste estrutural do gene *Vg* com outras sequências já publicadas e disponíveis no GenBank.

6. Revisão de Literatura

ALLSOPP, M. H.; DE LANGE, W. J.; VELDTMAN, R. Valuing insect pollination services with cost of replacement. **PLoS One** 3:e3128, 2008.

ASHBY, R.; FORÊT, S.; SEARLE, I.; MALESZKA, R. MicroRNAs in honey bee caste determination. **Scientific Reports**, 6, p.18794, 2016.

BAKER, M. E. Is Vitellogenin an ancestor of Apolipoprotein-B-100 of human low-density lipoprotein and human lipoprotein-lipase. **Biochemical Journal**, 255, p. 1057-1060, 1998.

BIENEFELD, K.; EHRHARDT, K.; REINHARDT, F. Genetic evaluation in the honey bee considering queen and worker effects - a BLUP - animal model approach. **Apidologie**, 38, p. 77-85, 2007.

BIENEFELD, K.; PIRCHNER, F. Heritabilities for several colony traits in the honeybee (*Apis mellifera carnica*). **Apidologie**, 21(3), p. 175-183, 1990.

- BÖRGER, K. Zur Selektion von Bienenvölkern auf Sammelleistung. **Zeitschrift für Bienenforschung**, 9, p. 545–564, 1969.
- BRASCAMP, E.; BIJMA, P. Methods to estimate breeding values in honey bees. **Genetics, Selection, Evolution**, 46(1), p. 53, 2014.
- CHEVALET, C.; CORNUET, J. M. Étude théorique sur la selection du caractère production de miel chez l'abeille. I. Modèle génétique et statistique. **Apidologie**, 18(3), 39-65p, 1982.
- COBEY, S.; SHEPPARD, S.; TARPY, D. Status of breeding practices and genetic diversity in domestic US honey bees. **Honey bee colony health: Challenges and Sustainable Solutions**. CRC, Boca Raton, Florida, p. 39-49, 2012.
- COLLINS, A.; RINDERER, T.; HARBO, J.; BROWN, M. Heritabilities and correlations for several characters in the honey bee. **Journal of Heredity**, 74, p. 135-140, 1984.
- COMEX. Comex Stat. Disponível em: comexstat.mdic.gov.br. Acesso em: 10 nov. 2020.
- COSTA-MAIA, F. M. Aspectos genéticos da produção de mel e comportamento higiênico em abelhas *Apis mellifera* africanizadas. **Tese** (Doutorado em Zootecnia) – Programa de Pós Graduação em Zootecnia, Universidade Estadual de Maringá, Maringá. 77p, 2009.
- COSTA-MAIA, F. M.; LINO, D. A. Melhoramento genético em abelhas *Apis mellifera* africanizadas: algumas questões decisivas. **Sistemas de produção agropecuária**. Curitiba: UTFPR, 22, p. 434-449, 2009.
- COSTA-MAIA, F. M.; MARTINS, E. N.; RODRIGUES, M. C. et al. Abstract: Genetic parameters for reproductive traits of Africanized drones *Apis mellifera* L. (Apidae). 66th **Annual Meeting of the European Federation of Animal Science**, Warsaw, Poland, 2015.
- COSTA-MAIA, F. M.; TOLEDO, V. A.; MARTINS, E. N. et al. Estimates of covariance components for hygienic behavior in Africanized honeybees (*Apis mellifera*). **Revista Brasileira de Zootecnia**, 40 (9), p. 1909-1916, 2011.
- COSTA, F.M. Estimativas de parâmetros genéticos e fenotípicos para peso e medidas morfométricas em rainhas *Apis mellifera* africanizadas. **Dissertação** (Mestrado em Zootecnia) – Universidade Estadual de

- Maringá, Universidade Estadual de Maringá, Maringá. 39p, 2005.
- CROW, J. F.; ROBERTS, W. C. Inbreeding and homozygosity in bees. **Genetics**, 35, p. 612–621, 1950.
- DELANEY, D.; Keller J.; CAREN, J.; TARPY, D. The physical, insemination, and reproductive quality of honey bee queens (*Apis mellifera* L.). **Apidologie**, Springer Verlag, 42 (1), p. 1-13, 2011.
- DE FIGUEIREDO J., SANTANA, H. et al. Identifying successful strategies for honey value chains in Brazil: a conjoint study. **British Food Journal**, 2016.
- DOS SANTOS, C. F.; OTESBELGUE, A.; BLOCHTEIN, B. The dilemma of agricultural pollination in Brazil: Beekeeping growth and insecticide use. **PloS One**, 13 (7), 2018.
- EL-BANBY, M. Abschätzung der Heritabilität und der genetischen Korrelationen der Brutaufzucht und der Honigproduktion bei der Honigbiene. Int. Bienenzüchter Kongr. **Apimondia Verlag Bukarest**, 21, p. 551–552, 1967.
- FALCONER, D. S. Introdução a genética quantitativa. Viçosa: UFV. **Imprensa Universitária**, 279p, 1987.
- FAO – FOOD AND AGRICULTURE ORGANIZATION. Data, Production. Disponível em: faostat.fao.org. Acesso em: 10 de nov. 2020.
- FAQUINELLO, P. A valiação genética em abelhas *Apis mellifera* africanizadas para produção de geléia real. **Dissertação** (Mestrado em Zootecnia) – UEM, Universidade Estadual de Maringá, Maringá. 54p, 2007.
- FUCHS, S.; SCHADE, V. Lower performance in honey bee colonies of uniform paternity. **Apidologie**, 24, p. 155-168, 1994.
- GRAMACHO, K. P. Considerações sobre o melhoramento de abelhas com base no comportamento higiênico. In: **Congresso Brasileiro De Apicultura**. Anais de Congresso, Natal, 15, 2004.
- HENDERSON, C. R. Best linear unbiased estimation and prediction under a selection model, **Biometrics**, 31 (2), p. 423–447, 1975.
- HOOPINGARNER, R; FARRAR, L. Genetic control of size in queen honey bees. **Journal of Economic Entomology**, 52. p. 547-548, 1959.
- JACKSON, J. T.; TARPY, D. R.; FAHRBACH, S. E. Histological Estimates of Ovariole Number in Honey Bee Queens, *Apis mellifera*, Reveal Lack of

- Correlation with other Queen Quality Measures. **Journal of Insect Science**, 11, (82), p. 82, 2011.
- KENT, C.; ISSA, A.; BUNTING, A. C.; ZAYED, A. Adaptive evolution of a key gene affecting queen and worker traits in the honey bee, *Apis mellifera*. **Molecular Ecology**, 20, 5226–5235, 2011.
- KERR, W. E. Introdução de abelhas africanas no Brasil. **Brasil Apicola**, 3, p. 211-213, 1957.
- KERR, W. E. The history of introduction of African bees to Brazil. **South African Bee Journal**. 39, p. 3-5, 1967.
- LAILAW, H. H.; PAGE, R. E. Polyandry in honey bees (*Apis mellifera* L.): Sperm utilization and intracolony genetic relationship. **Genetics**, 108, p.985-997, 1984.
- LE CONTE, Y.; MOHAMMEDI, A.; RODINSON, G. E. Primer effects of a brood pheromone on honeybee behavioral development. **Proceedings of the Royal Society of London**, 268, p. 163-164, 2001.
- LU, C. et al. Mitochondrial Dysfunction: A Plausible Pathway for Honeybee Colony Collapse Disorder (CCD). **Environmental Science & Technology Letters** 7.4, 254-258, 2020.
- LOPES, P. S. Teoria do Melhoramento Animal. Belo Horizonte: **FEPMVZ-Editora**, 118p, 2005.
- MALKOV V.; SEDYKH A. Selection of bee colonies for productivity under relatively poor honey flow conditions. **Apiacta**, 15, p. 66-69, 1980.
- MARTINS, J. Aspectos genéticos de características morfológicas e reprodutivas de rainhas *Apis mellifera* L. (Hymenoptera: Apidae) africanizadas. 89p. **Dissertação** (Mestrado em Zootecnia) Programa de Pós Graduação em Zootecnia - Universidade Tecnológica Federal do Paraná, Dois Vizinhos, 2014.
- MILNE, C. P. An estimate of the heritability of the corbicular area of the honeybee. **Journal of Apicultural Research**, 24, p.137-139, 1985.
- MILNE, C. P. Jr.; HELLMICH, R. L. II.; PRIES, K. J. Corbicular size in workers from honeybee lines selected for high or low pollen hoarding. **Journal of Apicultural Research**, 25(1), 50-52, 1986.
- MILNE, C. P. Jr.; PRIES, K. J. Honeybees with larger corbiculae carry larger

- pollen pellets. **Journal of Apicultural Research**, 25(1), 53-54, 1986.
- MILNE, C. P.; PRIES, K. J. Honeybee corbicular size and honey production. **Journal of Apicultural Research**, 23, p. 11-14, 1984.
- MORITZ, R. F. A. Comparison of within-family and mass selection in honeybee populations. **Journal of Apicultural Research**, 25, p. 146-453, 1986.
- MORITZ, R. F. A., SOUTHWICK, E. E. Bees as superorganisms: an evolutionary reality. **Springer Science e Business Media**, 395p, 1992
- MOSTAJERAN, M; EDRISS M. A.; EBADI, R. et al. Heritability estimates of morphological characters and honey yield of honeybee colonies in Isfahan. **Journal of Science e Technology of Agriculture and Natural Resources**, 4, p. 119-126, 2000.
- MUTTI, N. S.; DOLEZAL, A. G.; WOLSCHIN, F.; MUTTI, J. S.; GILL, K. S.; AMDAM, G. V. IRS and TOR nutrient-signaling pathways act via juvenile hormone to influence honey bee caste fate. **Journal of Experimental Biology**, 214, p. 3977-3984, 2011.
- NOGUEIRA-COUTO, R. H.; COUTO, L. A. Apicultura: manejo e produtos. 3. ed. **Jaboticabal: FUNEP**, 2006.
- NUNES, F. M.; IHLE, K. E.; MUTTI, N. S.; SIMÕES, Z. L.; & AMDAM, G. V. The gene vitellogenin affects microRNA regulation in honey bee (*Apis mellifera*) fat body and brain. **Journal of Experimental Biology**, 216 (19), p. 3724-3732, 2013.
- OLDROYD, B.; MORAN, C.; NICHOLAS F. Diallel crosses of honeybees. II A note presenting an estimate of the heritability of honey production under Australian conditions. **Australian Journal of Agricultural Research**, 38, p. 651-654, 1987.
- PADILHA, A. H; SATTTLER A.; COBUCI, J. et al. Genetic parameters for five traits in Africanized honeybees using Bayesian inference. **Genetics and Molecular Biology**, 36 (2), p. 207-213, 2013.
- PAGE, R. E.; LAIDLAW, H. H. Closed population honeybee breeding. 2. Comparative methods of stock maintenance and selective breeding. **Journal of Apicultural Research**, 21(1), p. 38-44, 1982.
- PAGE, R. E.; LAIDLAW, H. H. Honey bee genetics and breeding. In: GRAHAM, J.M. (Ed.) **The hive and the honey bee**. Illinois: Dadant and Sons, 253-267p., 1997.

- PEREIRA, F. M.; LOPES, M. T. R.; CAMARGO, R. C. R.; VILELA, S. L. O. Produção de mel. **Sistema de Produção. EMBRAPA**, Meio Norte, julho, 129p. 2003.
- PIRCHNER, F.; RUTTNER, F. Erbliche unterschiede zwizchen entragseigenschaften von bienen. **Internacional Apiculture Congress**, 2, p. 501-506, 1962.
- PIRCHNER, F.; RUTTNER, F.; RUTTNER, H. Erbliche Unterschiede zwischen Ertragseigenschaften von Bienen. **XI International Congress Entomology**, Vienna, p. 510- 516, 1960.
- PIULACHS, M. D.; GUIDUGLI, K. R.; BARCHUK, A. R. et al. The vitellogenin of the honey bee, *Apis mellifera*: Structural analysis of the cDNA and expression studies. **Insect Biochemistry and Molecular Biology**, 33(4), p. 459–465, 2003.
- POLHEMUS, M. S.; LUSH, J. L.; ROTHENBUHLER, W. C. Mating systems in honey bees. **Journal of Heredity**, 41, p. 151-154, 1950.
- QIN, X.; EVANS, J. D.; ARONSTEIN, K. A.; MURRAY, K. D.; WEINSTOCK, G. M. Genome sequences of the honey bee pathogens *Paenibacillus larvae* and *Ascosphaera apis*. **Insect Molecular Biology**, 15, p. 715–718, 2006.
- R Development Core Team (2013) R: a language and environment for statistical computing. Vienna, Austria: **R Foundation for Statistical Computing**, 2013. Disponível em: <<http://www.R-project.org>> Acesso em 08 mar. 2017.
- RACHINSKY, A.; STRAMBI, C.; STRAMBI, A. et al. Caste and metamorphosis: hemolymph titers of juvenile hormone and ecdysteroids in last instar honey bee larvae. **General and Comparative Endocrinology**, 79, p. 31–38, 1990.
- RANGEL, R. Mel brasileiro conquista o mercado externo. **Revista Inovação em Pauta**, 10, 56-61, 2010.
- REHDER, C. P. Apicultura Sustentável: As fronteiras da apicultura brasileira. Rio Claro, São Paulo 2013. Disponível em: <<http://www.abemel.com.br>>. Acesso em: 17 mar. 2017.
- RINDERER, T. E. Measuring the heritability of characters of honeybees. **Journal of Apicultural Research**, 16(1), p. 95-98, 1977.
- RINDERER, T. E.; COLLINS, A. M. Bee Genetics and Breeding. **Elsevier**.

- Academic Press: London, p. 391–413, 1986.
- RODRIGUES, M. C. Genetic parameters for morphometrics traits of africanized honeybee drones. **Dissertação** (Mestrado em Zootecnia – Melhoramento Genético Animal) – Programa de Pós Graduação em Zootecnia, Universidade Tecnológica Federal do Paraná, Dois Vizinhos. 85p, 2016.
- RUDEN, D. M.; CINGOLANI, P. E.; SEN, A. et al. Epigenetics as an answer to Darwin's "special difficulty," part 2: Natural selection of metastable epialleles in honeybee castes. *Frontiers in Genetics*, 6, p. 60, 2015.
- SADD, B. M.; BARRIBEAU, S. M.; BLOCH, G. et al. The genomes of two key bumblebee species with primitive eusocial organization. **Genome Biology**, 16(1), p. 76, 2015.
- SANGER, F.; NICKLEN, S.; COULSON, A. R. DNA sequencing with chain-terminating inhibitors. **Proceedings of the National Academy of Sciences**. U.S.A, 74(12), p. 5463-5467, 1977.
- SCHMIDT-CAPELLA, I. C.; HARTFELDER, K. Juvenile hormone effect on DNA synthesis and apoptosis in caste-specific differentiation of the larval honey bee (*Apis mellifera*) ovary. **Journal of Insect Physiology**, 44, p. 385–391, 1998.
- SILVEIRA-JÚNIOR, C. E. A., et al. Evaluation of honey quality of Northeast of Brazil: botanical origin and heavy metals content. **Journal of Apicultural Research**, 1-16, 2020.
- SOARES, A. E. Abelhas africanizadas no Brasil: do impacto inicial às grandes transformações. In: **Reunião anual da sociedade brasileira para o progresso da Ciência**, São Luís. Anais do congresso, 64, 2012.
- SOLLER, M.; BAR-COHEN, R. Some observations on the heritability and genetic correlation between honey production and brood area in the honey bee. **Journal of Apicultural Research**, 6(1), p. 37-43, 1967.
- TANAKA, E; HARTFELDER, K. The initial stages of oogenesis and their relation to differential fertility in the honey bee (*Apis mellifera*) castes. **Arthropod Structure Development**, 33 p. 431–442, 2004.
- TARPY, D. R.; HATCH, S.; FLETCHER, J. C. The influence of queen age and quality during queen replacement in honeybee colonies. **Animal Behaviour**, 59(1), p. 97-101, 2000.
- VESELY, V.; SILER, R. Die V erwenungsmöglichkeit der quantitativen und

- Populationsgenetik bei der Rassezucht von Bienen. **Vorträge des 19 Apimondia- Kongress 2**, p. 534–536, 1964.
- WILLAM, A.; EßL, A. Schätzung von Populationsparametern für verschiedene Merkmale bei der Honigbiene (*Apis mellifera carnica*). **Apidologie**, 24, p. 355–364, 1993.
- WILLHAM, R. L. The covariance between relatives for characters composed of components contributed by related individuals. **Biometrics**, 19, 18-27p, 1963.
- WINSTON, M. L. A biologia da abelha. Tradução de Carlos Alberto Osowski. **Editora Magister: Porto Alegre**, 276p, 2003.
- ZAWILSKI, K. Genetic breeding morphological index of the honey bee. **Pr. i mater. Zootechn**, 5, 1974.
- ZHOU, X.; STEPHENS, M. Genome-wide efficient mixed-model analysis for association studies. **Nature Genetics**, 44, p. 821–824, 2012.

CHAPTER II

Why does vitellogenin matter? A review on behavior and reproductive biology of honeybees and other insects

O artigo a seguir está redigido de acordo com as normas para publicação na revista *Apidologie* para ser submetido como **Review Article**.

CHAPTER II - Why does vitellogenin matter? A review towards behavior and reproductive biology of honeybees and other insects

Abstract

Vitellogenin (Vg) is an egg-yolk precursor, shielding effects in egg-laying ability and queens performance. However, its functions go beyond reproduction, its titres vary between castes and affect reproductive social status, nursing, foraging, longevity, somatic maintenance, and immunity. Vg is synthesized in the fat body of both queens and workers and the ovaries of queens. The hypothesis of reproduction-related functions is supported by studies demonstrating that its reproductive potential, assessed by the levels of vitellogenin, gene expression, and pathways, is connected to the ovarian development and task performance. The present review showcases the critical role of vitellogenin in behavior, reproduction and how it can shape and affect the reproductive biology and performance of honey bees.

Introduction

Vitellogenin is a hallmark of pleiotropy and its study comprises two central questions: the first concerned with the regulatory functions that go behind behavior, immunity, longevity, social organization and reproduction and the second with the vitellogenin structural gene sequences as a tool for phylogenetic modelling in *Apis mellifera*. An understanding of the factors associated with the specific role of this gene and protein requires a deep reading and comparisons with its role in other egg-laying animals including insects and vertebrates such as fish and birds. The honeybee, *Apis mellifera*,

is the forefront genetic and physiological model system for unravelling the social behaviour and polyethism in insects.

Most of the previous studies in social insects suggested that reproductive division of labor between queens and workers, might be triggered by modifications in the key regulatory pathways of reproduction and life history which are the insulin/insulin like signalling and hormone-like protein called vitellogenin. Besides, vitellogenin does not only form egg-yolk, but plays multiple roles in life-span regulation such as (i) provide nutrients; (ii) social organization; (iii) hormonal dynamics regulation; (iv) pattern recognition; (v) antibacterial/bactericidal activity; (vi) opsonization; (vii) pathogens aggregation; (viii) hemagglutination; (ix) antiviral defense and (x) antioxidant activity. Also, vitellogenin and its correspondent hormones have been extensively studied in terms of being a candidate gene that have been associated with worker sterility (Barchuk et al. 2002; Piulachs et al. 2003; Amdam et al., 2003; Seehuus et al. 2006; Corona et al. 2007; Seehuus et al. 2007; Wang et al., 2010; Ament et al. 2011; Kent et al. 2011; Wheeler et al. 2013) and with ovary size and state (Grozinger et al. 2007; Guidugli-Lazzarini et al. 2008; Brito et al. 2010; Chen et al. 2012; Awde et al. 2020). Excellent book chapters and reviews have covered extensively (Kent et al. 2011; Tufail and Takeda, 2008; Guidugli et al. 2005; Awde et al. 2020). Here, we describe theoretical studies that can shed light and illustrate vitellogenin's pleiotropic characteristics throughout, targeting its role on reproduction.

Characterization of Vg

Vitellogenin is a phospholipidglycoprotein (Wheeler and Kawooya, 1990) that acts as the primary egg-yolk precursor protein in oviparous animals,

and so has been described as a major reproductive protein (Li et al., 2008). Its primary function is to bind and subsequently transport maternal nutrients as lipids, carbohydrates, metals (Mg, Ca, and Zn), and phosphorous to the oocyte (Falchuk and Montorzi, 2001), where it is absorbed into tissue via receptor-mediated endocytosis (Tufail and Takeda, 2008). This way, vitellogenin is responsible for providing the resources towards embryogenesis. *Vg* is part of a superfamily of genes, recorded as very ancient, dating back 700 million years (Baker, 1988), is believed to be the most ancestral and consequently, the oldest of these proteins (Hayward et al., 2010).

The superfamily of genes where *Vg* belongs, is known as large lipid transport proteins (LLTPs), including key mammalian transport proteins such as the large subunit of microsomal triglyceride transfer protein (MTP) (Babin et al., 1999), and the transporter protein of cholesterol - apolipoprotein B (apoB-100) (Babin and Gibbons, 2009). These proteins function as inflammation suppressors, immunomodulators, and blood coagulants. In the specific case of vitellogenin, its most described function is related to the transport of protein stems and its ability to bind to lipids, which arises from its biochemical structure.

The protein is described to be cleaved at various sites in different species, and several structural domains are well conserved, for both the genomic sequence and structural level, across taxa (Mann et al., 1999). One such domain is the N-terminus or N-sheet, which harbors the lipid-binding cavity (Anderson et al. 1998; Roth et al., 2013). The other segment is the α -helical domain, which contains a lipophilic cavity implicated in binding to various ligands (Figure 1). The α -helical domain is believed to facilitate vitellogenin's anti-inflammatory functions (Salmela et al., 2016).

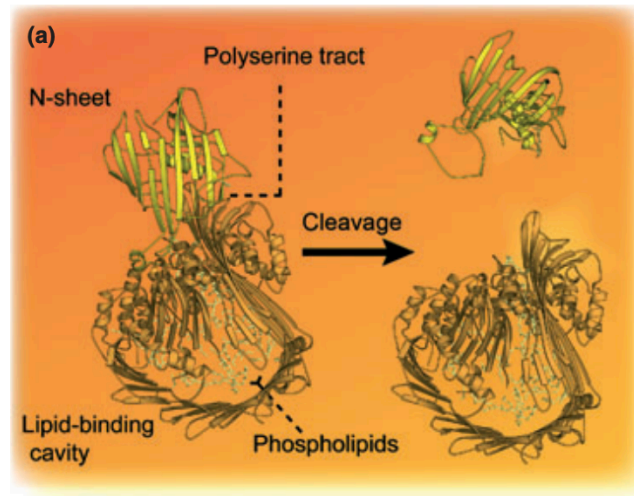


Figure 1. Illustration of the vitellogenin protein based on the lamprey lipovitellin X-ray structure by Anderson et al. (1998). The conserved N-sheet can get cleaved from the major lipid binding cavity, forming two independent fragments.

How it acts

In insects, vitellogenin is synthesized by the ovary and the fat body. The latter is an organ with functions in homeostasis, immunity, protein synthesis, and nutrient storage. The fat body is primarily located in the abdominal body compartment and is analogous to vertebrate liver and adipose tissues. During the female reproductive cycle, the rate of production of vitellogenins by the fat body is typically coordinated with titers of ecdysteroids and juvenile hormones (JHs) (Awde et al., 2020). Often, ecdysteroid signalling is required for fat body cells to achieve competence to synthesize vitellogenin at elevated levels, whereas JH modulates rates of production per se (Raikhel et al., 2005; Kapheim and Johnson, 2017).

Vitellogenin synthesis, ovarian uptake, and oocyte maturation, furthermore, are processes highly conditional on sufficient nutrient availability. Nutritional status is sensed, at least in part, by signalling through insulin/insulin-like pathways and by JHs and target of rapamycin (TOR, a serine/threonine kinase)-dependent mechanisms (Murphy et al., 2009; Nilsen et al., 2011). Nutritional resources to complete vitellogenesis were found to be present in pupal females of *Oestrus ovis* (Diptera: Oestridae), affecting its gonotrophic development, or as storage proteins that carry over from pupal development (for instance, in *Drosophila*) (Cepeda-Palacios and Scholl, 1999). Nowadays, the concepts of vitellogenin studies development has shifted far beyond egg development and/or lipid transport.

Nelson et al. (2007), in a pioneering research have combined a multitude of unknown functions for vitellogenin, including its role in social ontogeny, behavior (Amdam et al., 2006), life span (Corona et al., 2007; Havukainen et al., 2013), and immunity (Amdam et al., 2004; Brandt et al., 2005; Salmela et al., 2015). A single honeybee vitellogenin protein was first detected by Engels (1972). It was described as a ~180-kDa protein encoded by a single gene - Vg (Piulachs et al. 2003), but is now known to be cleaved to form a 150-kDa and 40-kDa subunit (Havukainen et al., 2011). The 40-kDa subunit contains the N-sheet and the receptor binding structure and is found primarily in the fat body. While the main unit, 150-kDa subunit, contains the α -helical domain and is found in queen hemolymph and ovaries (Seehus et al., 2007) as well as in workers' head in the hypopharyngeal glands (Amdam et al., 2003). The functional 3D structure has a predicted model approach outlined in Havukainen et al. (2011) study. As in solitary insects, honeybee vitellogenin synthesis is

conditional on nutritional status and on the availability and quality of pollen in particular (Bitondi and Simões, 1996).

Vitellogenin and its key role in reproduction and life cycle

It is not surprising to find high levels of vitellogenin circulating in queen hemolymph, but its discovery in the functionally sterile female workers and the male drones was somehow enigmatic (Trenczek et al., 1989; Engels et al. 1990). Circulating vitellogenin titers is low in drones (100- to 1000-fold less than in queens), but levels can be surprisingly high in workers (up to the same concentrations as in queens). Worker honeybees have greatly reduced ovaries that, in principle, can nourish a limited number of eggs. Nonetheless, vitellogenin titers vary greatly through the life-cycle progression of worker bees and queens (Figure 2), and these temporal dynamics fall into predictable patterns that correlate with behavioral transitions that characterize worker ontogeny (Amdam et al. 2005).

Following Figure 2, in queens (a–b), vitellogenin (orange) as a precursor that may also enhance longevity through effects on metabolism and oxidative stress resistance. Taking queens to survive up to 4 years as they maintain constant high blood levels of vitellogenin, even during periods when they do not lay eggs (Seehuus et al. 2006). In workers (c–d), vitellogenin influences social behaviour. Nurse bees, that care for larvae, have high vitellogenin levels and use vitellogenin-derived products in larval food. When the bees' blood levels of vitellogenin decline 2–3 weeks later, the workers switch from caregiving to risky activities as foraging. Vg regulation and changes result is a division of labour between nest and field activities (Nelson et al. 2007).

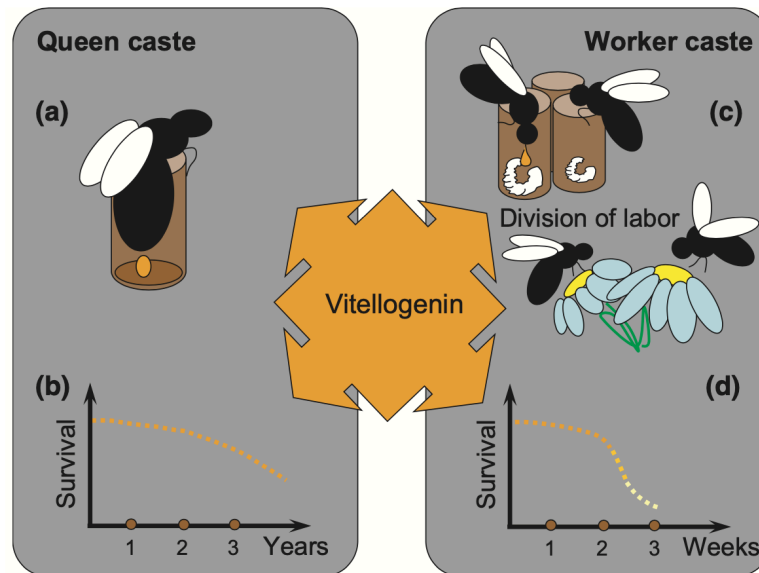


Figure 2. Representation of socially pleiotropic functions of vitellogenin in the honey bee *Apis mellifera*, from Amdam et al. (2005).

The high vitellogenin levels of nest workers have been physiologically connected to increased oxidative stress resistance and immunity (Seehuus et al. 2006). Workers ontogeny changes with the annual cycle of the colony. Seeley (1982), verified that when ambient conditions favor flight activity and foraging, workers go through an age-related behavioral sequence. After emergence until the first two week, they primarily stay in the central nest and perform tasks associated with brood care such as feeding, fanning, and warming. Later, they perform labor outside the colony as foragers, collecting nectar and pollen, as well as water and propolis.

Vitellogenin synthesis changes during the life cycle of an individual, being negligible at the time of adult emergence, increasing rapidly within 2–3 days, ending up being the predominant hemolymph protein in the mature nurse bees: 30–50% of the protein total at 5–15 days of age, sufficient for an ovary to produce 30–100 eggs daily (Fluri et al., 1982; Engels et al., 1990). During

foraging, vitellogenin synthesis and circulating titers decline, and subsequently levels are negligible in foragers (Ihle et al., 2010). The differences in vitellogenin levels in different tasks phases of workers are still long enigmatic.

The phenomenon, as well as the presence of vitellogenin in the male drones, was first rejected as to have pleiotropic side effect or evolutionary mechanism reflecting the extreme strains on synthesis in reproductive queens (Engels, 1974; Engels et al., 1990). These findings suggesting that selection favored loss of vitellogenin expression in workers and drones. It is important to highlight the constraints inherent to the causal genetic or epigenetic networks that prohibited the evolution of efficient sex or caste-specific suppression combined with ample expression in the queens. In other hand, the first argument considered the pattern of dynamic expression in workers as a pleiotropic consequence of JH signalling. This signalling was then proposed to primarily modulate the activity of the hypopharyngeal glands - where royal jelly is produced.

Remaining to the conserved role of JH in the main pathways of insect reproduction, endocrine regulation of workers' gland expression would inevitably affect rates on the synthesis of vitellogenin. Both cases show that vitellogenin produced by workers and drones is assumed not to have specific functions. Data indicating to substantial vitellogenin trade in workers thus, remained taken as evidence for an capacity of the fat body to recycle the protein as a turnoff strategy. Rutz and Lüscher (1974), were the first to propose the functional role of honeybee vitellogenin that could address the phenomenon of changing titers in workers. They suggested that vitellogenin synthesis was higher in nurse bees for the specific drive of transfer by mouth (trophallaxis) to

the queen. They argued that worker-produced vitellogenin could be utilized directly to support the very high egg-laying rate that is characteristic of queens.

Nurturing performed by workers, is indeed essential to the reproductive effectiveness and survival of honeybee queens, which are not able to digest pollen and thus are unable to obtain amino acid resources on their own (Crailsheim, 1992). However, Rutz and Lüscher abandoned their hypothesis after vitellogenin immune-electrophoresis failed to detect yolk protein in proteinaceous bee jelly. This jelly is the nutritious secretions that are synthesized by the hypopharyngeal glands of the workers. Jelly is transferred by trophallaxis, and it is essential to the sustenance of queens, young larvae, and all newly emerged adults (Naiem et al., 1999).

Engels et al. (1990), suggested that the vitellogenin synthesis in drones, workers, and also in queens that do not lay eggs (virgin and/or mature queens during winter) reinforced the role of vitellogenin in honeybee metabolism. As aforementioned, and supported by many researches, is it known that vitellogenin does not act alone, its action has a deep and dynamic relationship with the systemic hormone JH, in what is called a *Double Repressor Model*. The latter hormone is synthesized and secreted by the *corpora allata* complex, paired glands situated behind the insect brain.

During larval development, JH interacts with ecdysteroids to control between-instar molts, and in adults the two hormones work together to govern reproductive development and behavior (Hartfelder, 2000). The *Double Repressor Model* specified that, although JH could have important functions in worker behavioral regulation or phenotypic integration, the system was controlled by two repressors: vitellogenin (the internal repressor) and the signal

stemming from the foragers (the external repressor or inhibitor mentioned above). In mechanistic terms, the internal and external repressors were envisioned as competing for binding to repressor receptor molecules in the allato-regulatory neuroendocrine axis of the brain, with JH synthesis suppressed at a certain fractional occupation of these receptors. A worker with ample vitellogenin stores would be unlikely to abandon her nursing tasks. But, as her vitellogenin stores became depleted, or if the colony demography shifted toward a higher ratio of nurse bees to foragers, the probability of initiating foraging would increase. The behavioral shift from nursing to foraging would be reinforced by the increase in JH that was released by the decrease in vitellogenin.

The *Double Repressor Model* proposed a new regulatory architecture of division of labor by opening the black box of the individual bee and building a feedback loop between vitellogenin and JH. It generated several testable predictions, including that starvation would result in precocious foraging behavior (Schultz et al., 1998), because rates of vitellogenin synthesis and consumption would be affected by the reduced availability of amino acid sources. The resulting lower levels of vitellogenin, next, would cause individual bees to become more likely to forage.

Gene structure

Vitellogenin was identified in honey bees about 50 years ago and its reproductive role in queens was instantly recognized (Engels 1974). In workers its expression was initially seen as evolutionary baggage; Nowadays, with all the studies available, it is known to have pleiotropic functions of the protein, leading to affect social behavior in workers, expend the protein in larval food,

enhance stress resistance, immunity and survival in both workers and queens. Kent et al. 2011 study provided the first evidence to date of positive selection acting on *Vg*, as being a socially pleiotropic gene in social insects that affects on several fitness traits that are partly separated in queen and worker castes. In insects, some species have several vitellogenin gene copies that may accommodate caste-specific functions, but honey bees have only one vitellogenin.

As a well-known pleiotropic gene, it was expected to be difficult to accumulate selectable mutations, while retaining or increasing its fitness contribution to two or more traits. Based on this hypothesis, Kent et al. (2011) set out to test whether honey bee vitellogenin experiences this constraint. The authors sequenced vitellogenin and seven other genes in 41 honey bee workers from Africa, East- and West Europe, with known population sizes, structures and histories. Based on this information, it was expected that changes or differences in population sizes were misinterpreted as signatures of selection in the data. Also, using seven additional genes served as reference points that would be equally affected by issues of population dynamics and genetic drift.

The genes studied in parallel with vitellogenin were *Erk7*, that, similar to *Vg* are expressed in both workers and queens and have been proven to have effects in workers' behavior. Kent et al. (2011), based on the estimates of the high average pairwise nucleotide differences, linkage disequilibrium and skew in the allele variation and frequency spectrum, suggested recurrent positive selection acting on the *Vg* gene. Comparing the ratio of nucleotide substitutions, synonymous and to those that change amino acid sequence,

nonsynonymous, flagged relative rates of nonsynonymous to synonymous divergence as positive selection. The results of this study featured the gene by showing a high relative rate in comparisons between the four bee species of the dataset: the honey bee *Apis mellifera*, Asian honey bee *A. cerana*, giant honey bee *A. dorsata* and dwarf honey bee *A. florea*. Specific analyses of the gene revealed also significant linkage disequilibrium only for European populations, and not African.

At that date, this finding supported the hypothesis that changes in vitellogenin occurred to accommodate colony survival in colder climates during and after the prehistoric migrations of *A. mellifera* from Africa to Europe (Amdam et al. 2005; Seehuus et al. 2006). Overall, Kent et al. (2011) could assign an ongoing rate of adaptive protein evolution to vitellogenin than the seven reference genes and concluded that social pleiotropy does not constrain honey bee vitellogenin adaptation by selection. This positive selection on honey bee vitellogenin brought up the question of how genetic changes translate to the level of protein structure and function.

Structurally, Vg has a complex and large domain structure, with this in mind one question remains: Has this molecule, novel functions while retaining its conserved role in yolk production? To evidenciate this hypothesis Kent et al. (2011) discussed the 64 single nucleotide polymorphisms (SNPs) on their findings, that are unequally distributed in the vitellogenin sequence. Roughly dividing the protein in two parts, the N-terminal domain (N-sheet) is resistant to change, while the major lipid-binding cavity is scattered with SNP hotspots (Figure 9).

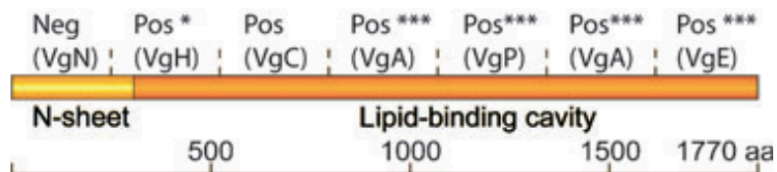


Figure 3. Adapted from Havukainen et al. and Kent et al. (2011), linear representation of the 1770 amino acid residues of Vg. The approximate locations of regions that are expected to be under positive (Pos) or negative (Neg) selection.

Besides Vg is a complex molecule, recent studies showed that there are degrees of freedom to sculpt parts of this molecule for novel functions while retaining its conserved role in yolk production. This hypothesis is supported by the replacement polymorphisms previously discovered in the gene (Kent et al. 2011) that were reported to significantly enriched in parts of the proteins involved in binding lipids. Which led to conclude that gene's structure may be linked with functions that affect performance and fitness. Also, there is evidence of clear signature of historical and positive selection acting on the honey bee's gene vitellogenin. For instance, the number of ovarioles is found to be a phenotypic indicator regarding queens reproductive performance (Delaney et al. 2011, Jackson et al. 2011).

Genetic variations could be responsible for what is observed as phenotypic variation of reproductive traits as the number of ovarioles. Kent et al. (2011) highlighted that the N-sheet contains the phylogenetically conserved, putative receptor-binding domain of vitellogenin is presumably important for uptake to the ovary (Li et al. 2003). This domain can be cleaved off the mature

protein in a process that appears to be regulated tissue specifically in honey bee workers (Havukainen et al. 2011). The biological significance of this dynamic remains not well known, but current evidence supports the conclusion of Kent et al. (2011) that the N-sheet is central to vitellogenin's conserved function in reproduction, while the remaining protein has been freer to evolve. Studies like this, promoted evidence of historical and adaptive evolution and how it was shaped by a key socially pleiotropic gene as *Vg*.

Vitellogenin gene knockdown

What happens if the vitellogenin gene suffers a knockdown? This question triggered Amdam et al. (2003) and Guidugli et al. (2005) to pursue their investigations on the gene *Vg* using RNAi. First developed in plants, RNAi has proven to be a valuable tool in the study of genetic networks in diverse species. RNAi allows for the targeted downregulation of gene expression, even in those organisms, such as honeybees, that pose challenges for reverse genetics (Amdam et al., 2003).

Guidugli et al. (2005) used RNAi to demonstrate that experimental suppression of vitellogenin expression was associated with a significant increase in JH hemolymph titer in different genetic and social backgrounds. The knockdown effect was robust in both European and African genotypes, and also in a normal colony setting versus small cages in which only dozens of worker bees make up the social environment (Guidugli et al., 2005). These results provided broad evidence for the proposed feedback loop between vitellogenin and JH. While the physiological plausibility of the Double Repressor model was confirmed by Guidugli et al. (2005).

Vitellogenin and its role in longevity and fertility

There are many measures that can serve as proxies for queen reproductive performance and/or quality. The most intuitive has been described to be standard morphological measures of individual adult insects, such as wet or dry weight, thorax width, head width, and wing lengths (Hatch et al., 1999; Gilley et al., 2003; Kahya et al., 2008), several of which are significantly correlated with queen reproductive success or fecundity (Woyke, 1971; Nelson and Gary, 1983; Costa-Maia et al., 2011). Vitellogenin has also been under the spotlight since is described as a potential indicator of fecundity for being the yolk precursor, mainly associated with egg production (Engels, 1974; Tanaka and Hartfelder, 2004). Queen's weight was positively correlated with Vg expression (Delaney et al., 2011) and, consequently, laying queens had significantly higher expression levels of Vg versus pre-laying queens. Vitellogenin is produced in the fat bodies, which is taken up by developing oocytes and is stimulated by mating (Kocher et al., 2010). Measures of Vg may be affected by normalizing expression levels using β -actin, which may also change as a function of weight or egg-laying status. Even still, the increased Vg levels in heavier queens is likely a consequence of larger fat bodies triggered by the process of mating (Kocher et al., 2008).

Normally, longevity is achieved at the expense of fertility, but queen honey bees do not show this tradeoff. Queens live longer and are fertile, while workers, from the same genome, are relatively short-lived and in normal conditions, sterile. To explore that hypothesis, Corona et al. (2007) and Seehuus et al. (2006) suggested, on the basis of their results from workers and

queens, that vitellogenin acts as an antioxidant and plays a role in queen longevity. Also, in Corona's findings, queens were also more resistant to oxidative stress than workers. Which supported and reinforced the hypothesis of caste-specific differences in *Vg* expression are involved in queen longevity. As such, *Vg* titers may serve as an effective proxy for queen reproductive quality and health (Delaney et al., 2011). In short, these studies led to suggest that conserved and species-specific mechanisms interact to regulate queen bee longevity without sacrificing fecundity.

Role on reproduction and behavior: other orders, other species

The next section is arranged to include a description of vitellogenin and its role on the reproduction in one species of each order mentioned above (Coleoptera, Hemiptera, Hymenoptera, Diptera, Lepidoptera), Zhang et al. (2019) unrooted consensus neighbour-joining tree displaying the evolutionary relationships among vitellogenin receptor proteins (VgR), as shown in Figure 3.

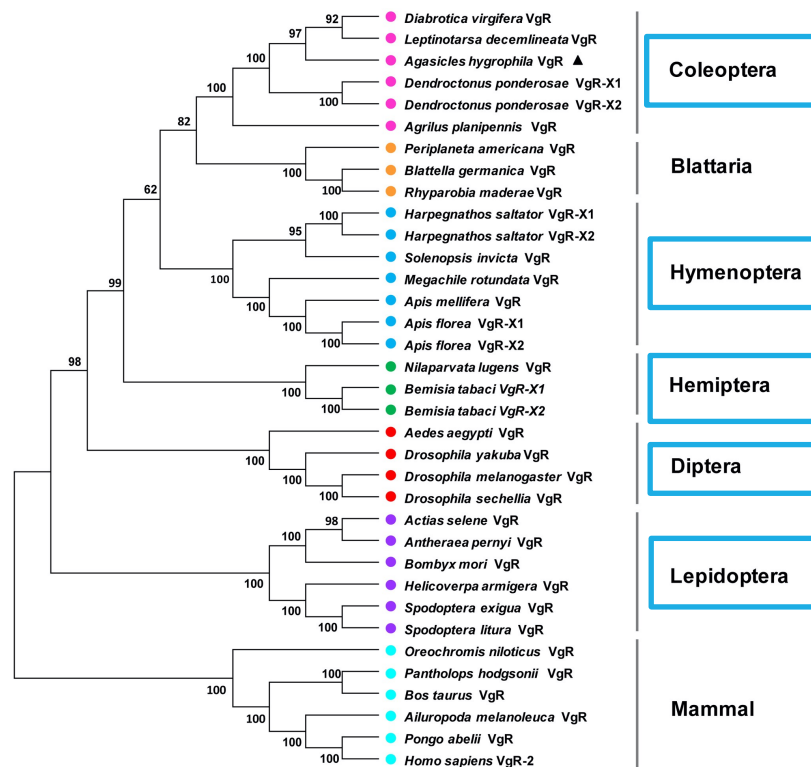


Figure 3. Phylogenetic tree, adapted from Zhang et al. (2019), generated with MEGA 5 using VgR amino acid sequences from insect species in the orders Coleoptera, Blattaria, Hymenoptera, Hemiptera, Diptera, Lepidoptera, and five mammal species. Orders on the blue boxes will be mentioned below with particular study cases.

Coleoptera

Zhang et al. (2019) studied the effect of an RNA interference mediated suppression of the vitellogenin receptors (VgRs) gene expression in adult *Agasicles hygrophila* (Coleoptera: Chrysomelidae). This species was introduced in many parts of the world as an effective biological control agent for the weed pest *Alternanthera philoxeroides*. Little is known about the reproduction of this important pest control insect. Vitellogenin receptors belong to the superfamily of low-density lipoprotein receptors (LDLRs) and was found to be directly expressed in ovarian tissues and also it is firstly transcribed in the newly-emerged females. One of the roles of VgRs is to regulate the absorption of yolk protein in insects. In this study, vitellogenin receptor gene (*VgR*) was sequenced and found to encode a predicted protein of 1,642 amino acids. The author concluded that RNA interference (RNAi)-mediated suppression of *VgR* gene expression in adult *A. hygrophila* females inhibited yolk protein deposition in the ovaries, shortened the ovariole, overall ovary size (Figure 4) and drastically reduced egg production, and ultimately led to a decrease in fecundity. Which reinforces that *VgR* is critical for transporting *Vg* into the oocytes and so, it plays an important role in *A. hygrophila* reproduction.

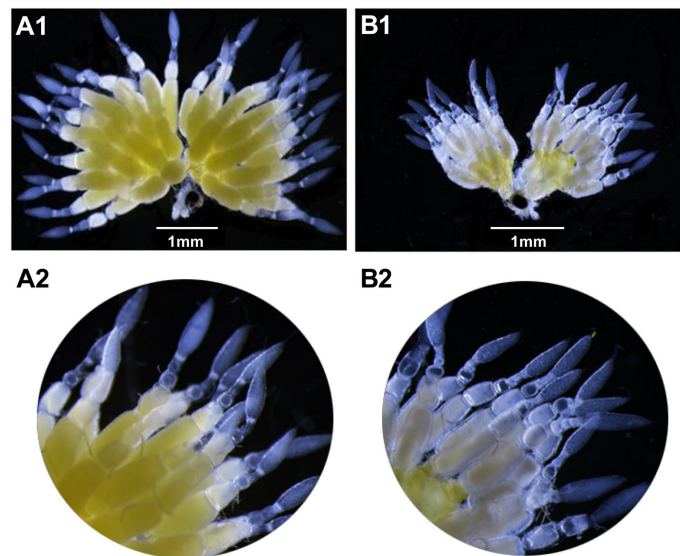


Figure 4. From Zhang et al. (2019), showing the effect of the injection of Double-stranded RNA interference (dsVgR) on *A. hygrophila* ovaries and ovarioles development and lengths. A1 and A2 shows an ovary that developed normally, in contrast with B1 and B2 that show an abnormal ovary that changed morphologically after injection of dsVgR.

Hemiptera

Shen Y et al. (2019) aiming to understand more about the brown planthopper, *Nilaparvata lugens*, the major pest for rice plants in Asia, carried out a study to investigate the effects of Vg in reproduction and development. In this study, the authors identified a Vg (*NIVg*) and two Vg-likes (*NIVg-like1* and *NIVg-like2*). Throughout phylogenetic analyses, showed that *NIVg-like1* and *NIVg-like2* are not clustered with the conventional insect Vgs associated with vitellogenesis. Temporo-spatial expression analyses have shown that the *NIVg* and *NIVg-like2* transcript levels increased significantly 24h after emergence and were primarily expressed in female adults. However, *NIVg-like1* was

expressed during all stages, and in both genders. Tissue-specific analyses showed that all three genes were most highly expressed in the fat body.

The injection of double-stranded RNA (ds*NIVg*) targeting *NIVg* showed that *NIVg* is essential not only for oocyte development but also for nymph development. The results illustrate the knockdown of *NIVg-like1* in female adults resulted in failure to hatch, death before eggshell emergence in 18% of offspring embryos and deformed and shorter individuals (Figure 5), which suggested that *NIVg-like1* plays an important role during late embryogenesis. Approximately 65% of eggs laid by females that were treated with double-stranded RNA targeting *NIVg-like2* failed to hatch, indicating that *NIVg-like2* plays a role in nutrition absorption during oocyte, or embryonic development.

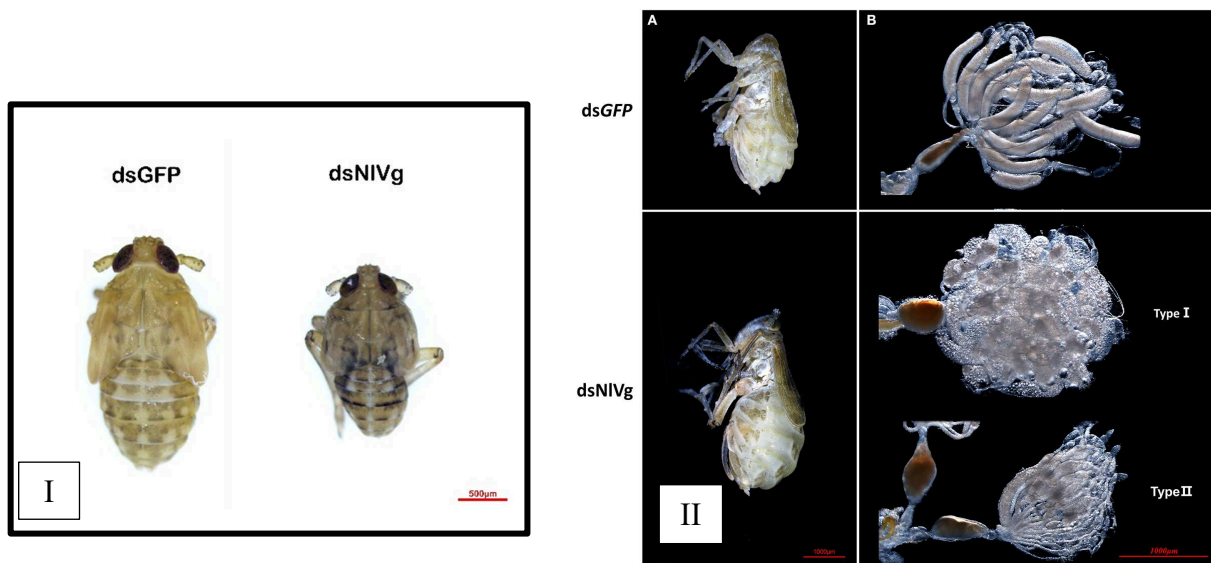


Figure 5. Adapted from Shen Y et al. (2019), show the effects of *NIVg* on nymphal development and oocytes. I. Injection of ds*NIVg* in nymphs (n = 100) caused a lethal phenotype, with shorter body shape compared to the control. II. Knockdown of *NIVg* leads to oocyte malformation. **(A)** Female at day 7 after

emergence ($n = 120$). ds*NIVg*-treated ($n = 120$) had larger abdomens compared to the control, with greatly stretched intersegmental membranes in the abdomen. **(B)** Oocytes derived from ds*GFP* and ds*NIVg*-treated female adults. At 9 days after emergence, oocytes in the ds*NIVg*-treated group were ball-shaped (type I) or filiform (type II) compared with the replete banana-shaped oocytes in the ds*GFP*-treated group.

Hymenoptera

Zhen et al. (2018) studied expression of this gene and reported important findings regarding gene transcriptional regulation associated with reproductive status of *Bombus lantschouensis*. The authors highlighted that to date (2018), there is a lack of studies regarding regulation of *Vg* transcription in bumblebees and its effects on reproduction. Zhen's et al. study not only describes vitellogenin's gene structure, expression pattern in this species but also mRNA expression patterns for various tissues during different reproductive stages of the queens. The authors, reported that *Vg* was found to be expressed at higher levels in reproductive queens than in virgins, also *Vg* was also expressed at the highest levels in the fat bodies of both virgin and reproductive queens in comparison with the other tested tissues: head, flight muscle and ovaries (Figure 6).

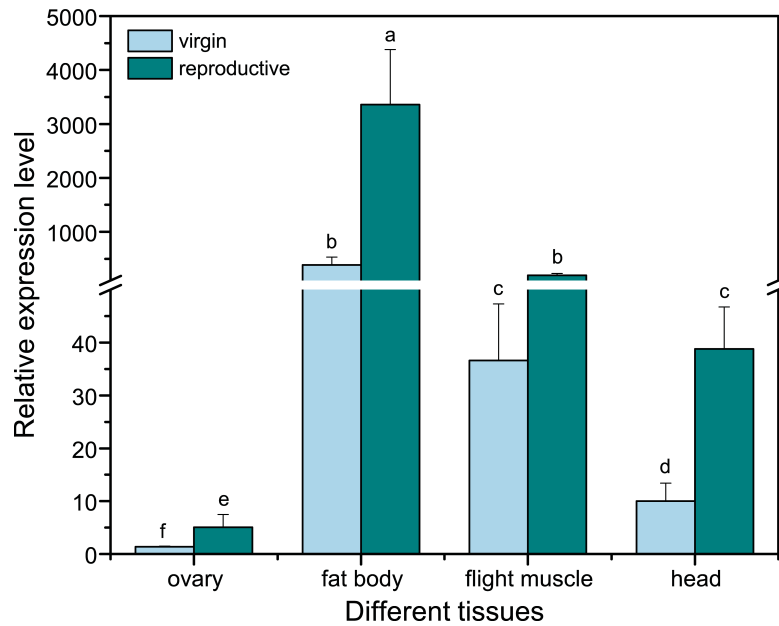


Figure 6. From Zhen et al. (2018), the graphic represents the output of an analysis of relative *Vg* expression in different tissues (ovary, fat body, flight muscle, head) of the bumblebee, *B. lantschouensis* of virgin and mated queens (colors). The lowercase letters above the error bars show that the expression levels were significantly different ($p < 0.05$) in the same tissue under different reproductive statuses.

It is known that transcriptional regulation of the *Vg* gene is regulated by hormones, including juvenile hormone (JH), ecdysteroids and some neuropeptides. Jedlička et al. (2016) study reported that JH was shown to increase *Vg* synthesis in reproductive bumblebee females by simultaneously monitoring the transcripts of methylfarnesoate epoxidase (MFE) and *Vg*. In some cases, *Vg* was uncoupled from or even negatively correlated with JH levels in *Bombus* species (Amsalem et al. 2014) while its transcription is

hormonally synchronized by transcription factors interacting with the upstream regulatory region of the *Vg* gene, mentioned before as N-sheet.

Diptera

Yolk proteins have been intensively studied in insects, specially in Diptera, due to its insights in dipteran evolution and adaptative mechanisms. *Aedes albopictus*, is a worldwide spread mosquito that is an epidemiologically important vector for the transmission of viral pathogens as yellow fever virus, dengue fever, Chikungunya fever as well as filarial nematodes and also capable to host Zika virus. This mosquito, as many others, turned into highly efficient vectors due to its great competence linked to a very successful reproductive strategy, where females need a vertebrate blood meal to develop large amount of eggs.

Even though the diseases that are highly spread are a huge concern, the molecular mechanisms and pathways regulating mosquito host-seeking behaviour, remain unknown. Dittmer et al. (2019) by knocking down the *Vg-2* gene via RNAi, restored host-seeking behaviour in females of *Ae. albopictus*. Firmly establishing that *Vg-2* gene expression has a pivotal role in regulating host-seeking behaviour in young females. The identification of a molecular mechanism regulating host-seeking behaviour in mosquitoes could pave the way for novel vector control strategies aiming to reduce the biting activity of mosquitoes. From an evolutionary perspective, this was the first demonstration of vitellogenin genes controlling feeding-related behaviours in non-social insects, while vitellogenins are known to regulate caste-specific foraging and brood-care behaviours in eusocial insects. Hence, Dittmer's research confirms

the key role of vitellogenin in controlling feeding-related behaviours in distantly related insect orders, suggesting that this function could be more ubiquitous than previously thought.

Lepidoptera

Plutella xylostella, known as diamondback moths are considered pests as they feed on the leaves of cruciferous crops. Vg is known to be crucial for yolk deposition and oocyte development. It has become a promising target to be studied for pest control. For this reason, Peng et al. (2020) studied the vitellogenin receptor (VgR), that belongs to the low-density lipoprotein receptor (LDLR) and plays a key role in Vg pathway and its consequent front of action, for a better understanding in how Vg is interloped with reproductive traits. The authors found that VgR was mainly expressed in female adults, more specifically in the ovary.

Through CRISPR/Cas9-mediated VgR knockout created a homozygous mutant of *P. xylostella* with 5-bp-nucleotide deletion in the VgR. The expression deficiency of VgR protein was detected in the ovaries and eggs of mutant individuals. Vg protein was still detected in the eggs of the mutant individuals, but with a decreased expression level. The authors, evidenced shorter ovarioles of newly emerged females as well as effected the size of the eggs, egg hatching rate, Vg transport, ovary and embryonic development (Figure 7 and 8). Understanding the molecular mechanism behind Vg in this species was highlighted to be a potential practical-strategy to better control of the *P. xylostella*, since it affects reproductive performance straightly.

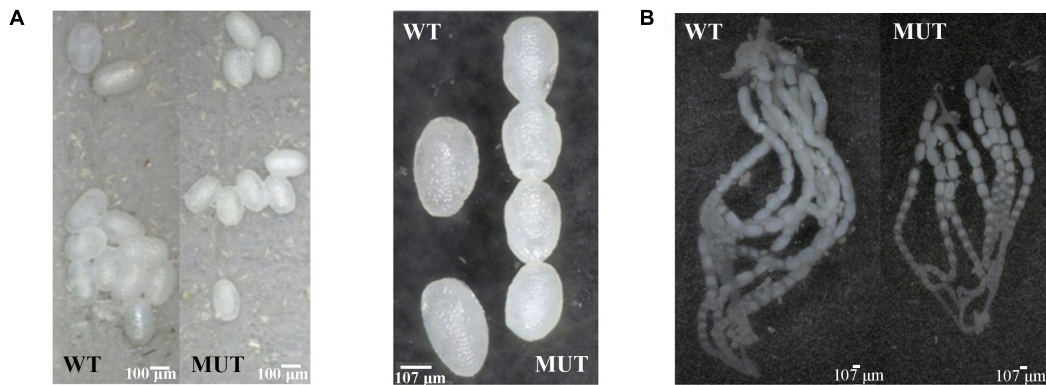


Figure 7. From Peng et al. (2020), the images reflect the effects of *VgR* knockout on ovary development and reproduction in *P. xylostella*. **(A)** The eggs were collected within 3 days of mating, and **(B)** the ovaries were dissected from newly emerged mutants (MUT) and wild types (WT) females.

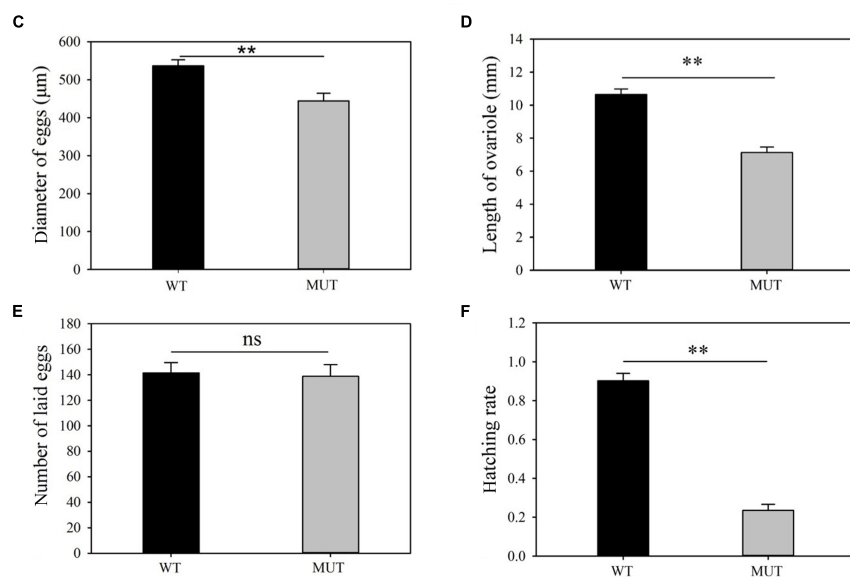


Figure 8. Results from Peng et al. (2020) **(C)** Diameter of eggs, **(D)** Length of ovariole, **(E)** Number of laid eggs, and **(F)** Hatching rate were analysed, respectively. The results were shown as mean ± SE. The asterisk ** above the bars represented significant difference ($P < 0.01$).

Prospects: Epigenetics affecting the expression of *Vg*

Both female castes are originated from fertilized eggs, however, changes in nutrition and the quantity and quality of food provided to young larvae stages, would trigger different epigenetically regulated development pathways (Maleszka et al. 2014). In Kucharski et al. (2008) study, the authors found that nutritional differences between females castes at larval level, can control their development via DNA methylation. Also, the amount of space in a comb cell, where larvae develop, can alter the DNA methylation level of the larval genome, and led to the process of caste differentiation (Shi et al. 2011). Alterations in gene regulation triggered by epigenetic mechanisms can cause divergent development paths, taking particular genes involved in signal transduction, gland development and metabolism of carbohydrates (Woodard et al. 2011). As per reported in Woyke's (1971) study, queens reared from young worker larvae shown smaller spermatheca and body size and fewer ovarioles. Rangel et al. (2012), concluded that colonies from queens grafted from older larvae, had significantly lower performance in egg-laying activity.

Furthermore, He et al. (2017), have identified genetic and epigenetic changes related to the age of larvae transplanted to queen cells to be reared as queens. In these authors findings, the main highlight was the identification of a candidate pathway for why grafting age changes queen ovariole number and spermatheca size. In this pathway, genes as MRJP-1, JH and *Vg* were found to be differentially expressed between different aged grafted queens. These aforementioned genes, are known to be deeply involved in the regulation of caste differentiation and longevity. Those findings shed a light on why queens from late-stage larvae, are undersized suggesting that *Vg* entering into these

epigenetic factors, plays a role in regulatory networks associated with the complex reproductive traits in honeybees.

Conclusion

There is no doubt that *Vg* is deeply linked to fecundity of honeybees: the great hallmark is its expression that is proved to be higher in the reproductive queens in comparison with sterile workers (Barchuk et al. 2002; Amdam et al. 2003; Corona et al. 2007), and circulating *Vg* titres match queen egg-laying rate (Engels 1974). *Vg* also affects lifespan and oxidative stress resistance in both queens and workers (Amdam et al. 2005; Corona et al. 2007). In closing, vitellogenin and its correspondent gene (*Vg*) has a role to play when we are looking at queens performance, fecundity and fitness, but still in need of evaluations that contrasts with more detailed phenotypic data and genomic structure of the gene.

Overall, this gene does really matter since it gives insights in the historical evolution of the species, and so, it should be considered in further breeding approaches targeting specific traits such as ovaries development and fecundity. The understanding of the effects that polymorphisms can have in the lipid-binding cavity of vitellogenin and how it can modify ligand-binding properties, would help to explain how vitellogenin regulates behavior, and how it can be translated into adaptive and phenotypical differences.

References

- Amdam, G. V., et al. (2005) Higher vitellogenin concentrations in honey bee workers may be an adaptation to life in temperate climates. *Insectes Sociaux*, 52 (4), 316-319.
- Amdam, Gro V., et al. (2003) Disruption of vitellogenin gene function in adult honeybees by intra-abdominal injection of double-stranded RNA. *BMC Biotechnology*, 3 (1), 1-8.
- Amdam, Gro V., et al. (2004) Hormonal control of the yolk precursor vitellogenin regulates immune function and longevity in honeybees. *Experimental Gerontology*, 39 (5), 767-773.
- Amdam, Gro V., et al. (2006) Downregulation of vitellogenin gene activity increases the gustatory responsiveness of honey bee workers (*Apis mellifera*). *Behavioural Brain Research*, 169 (2), 201-205.
- Ament, S. A., et al. (2011) Mechanisms of stable lipid loss in a social insect. *Journal of Experimental Biology*, 214 (22) 3808-3821.
- Amsalem, E. et al. (2014) Exploring the role of juvenile hormone and vitellogenin in reproduction and social behavior in bumble bees. *BMC Evolutionary Biology*. 14.
- Anderson, T. A., et al. (1998) The structural basis of lipid interactions in lipovitellin, a soluble lipoprotein. *Structure* 6, 895-909.
- Andersson, P. L., et al. (1999) Assessment of PCBs and hydroxylated PCBs as potential xenoestrogens: in vitro studies based on MCF-7 cell proliferation and induction of vitellogenin in primary culture of rainbow trout hepatocytes. *Archives of Environmental Contamination and Toxicology*, 37 (2), 145-150.
- Awde, D. N., et al. (2020) Vitellogenin expression corresponds with reproductive status and caste in a primitively eusocial bee. *Journal of Insect Physiology*, 104113.
- Azevedo, D. O., et al., (2011) Temporal variation of vitellogenin synthesis in *Ectatomma tuberculatum* (Formicidae: Ectatomminae) workers. *Journal of Insect Physiology* 57, 972-977.

- Babin, P. J., et al., (1999) Apolipoprotein II/I, apolipoprotein B, vitellogenin, and microsomal triglyceride transfer protein genes are derived from a common ancestor. *Journal of Molecular Evolution*, 49 (1), 150-160.
- Babin, P. J., Gibbons, G. F. (2009) The evolution of plasma cholesterol: direct utility or a “spandrel” of hepatic lipid metabolism? *Progress in Lipid Research*, 48 (2), 73-91.
- Baker, M. E. (1988) Is vitellogenin an ancestor of apolipoprotein B-100 of human low-density lipoprotein and human lipoprotein lipase? *Biochemical Journal*, 255 (3), 1057-1060.
- Barchuk, A. R., et al., (2002) Effects of juvenile hormone and ecdysone on the timing of vitellogenin appearance in hemolymph of queen and worker pupae of *Apis mellifera*. *Journal of Insect Science*, 2.
- Bitondi, M. M. G., Paulino Simoes, Z. L. (1996) The relationship between level of pollen in the diet, vitellogenin and juvenile hormone titres in Africanized *Apis mellifera* workers. *Journal of Apicultural Research* 35 (1), 27-36.
- Brandt, B. W., et al. (2005) Shuttling between species for pathways of lifespan regulation: a central role for the vitellogenin gene family?. *Bioessays* 27 (3), 339-346.
- Brito, R. M., M. et al. (2010) Expression of genes related to reproduction and pollen foraging in honey bees (*Apis mellifera*) narcotized with carbon dioxide. *Insect Molecular Biology* 19 (4), 451-461.
- Cepeda-Palacios, R., Scholl, P. J. (1999) Gonotrophic development in *Oestrus ovis* (Diptera: Oestridae). *Journal of Medical Entomology* 36 (4), 435-440.
- Chen, Jun, et al. (2012) Crossveinless d is a vitellogenin-like lipoprotein that binds BMPs and HSPGs, and is required for normal BMP signaling in the *Drosophila* wing. *Development* 139 (12), 2170-2176.
- Corona, M, et al. (2007) Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *Proc Natl Acad Sci USA*, 104, 7128-7133.
- Crailsheim, K. (1990) The protein balance of the honey bee worker. *Apidologie*, 21 (5), 417-429.
- Delaney, D., et al., (2011) The physical, insemination, and reproductive quality of honey bee queens (*Apis mellifera* L.). *Apidologie*, 42 (1), 1-13.

- Dittmer, J. et al., (2019) Fat body–specific vitellogenin expression regulates host-seeking behaviour in the mosquito *Aedes albopictus*. *PLoS Biology* 17 (5).
- Engels, W. (1972) Quantitative untersuchungen zum dotterprotein-haushalt der honigbeine (*Apis mellifera*). *Arch. Entwicklungsmech. Org.*, 171, 5
- Engels, W. (1974) Occurrence and significance of vitellogenin in female castes of social Hymenoptera. *American Zoologist*, 14, 1229-1237.
- Engels, W. et al., (1990) Honey bee reproduction: vitellogenin and caste-specific regulation of fertility. In: Hoshi, M., Yamashita, O. (Eds.), *Advances in Invertebrate Reproduction*, vol. 5. Elsevier, Amsterdam, 495–502.
- Falchuk, K. H., Montorzi, M. (2001) Zinc physiology and biochemistry in oocytes and embryos. In *Zinc Biochemistry, Physiology, and Homeostasis*, Springer, Dordrecht, 199-209.
- Flatt, T., et al., (2005) Hormonal pleiotropy and the juvenile hormone regulation of *Drosophila* development and life history. *Bioessays*, 27, 999-1010.
- Fluri, P., et al., (1977) Juvenile hormone, vitellogenin and haemocyte composition in winter worker honeybees (*Apis mellifera*). *Experientia*, 33(9), 1240-1241.
- Fluri, P., et al., (1982) Changes in weight of the pharyngeal gland and haemolymph titres of juvenile hormone, protein and vitellogenin in worker honey bees. *Journal of Insect Physiology*, 28(1), 61-68.
- Grozinger, C. M., et al. (2007) Genome-wide analysis reveals differences in brain gene expression patterns associated with caste and reproductive status in honey bees (*Apis mellifera*). *Molecular Ecology* 16 (22), 4837-4848.
- Guidugli-Lazzarini, K. R., et al. (2008) Expression analysis of putative vitellogenin and lipophorin receptors in honey bee (*Apis mellifera* L.) queens and workers. *Journal of Insect Physiology* 54 (7), 1138-1147.
- Guidugli, K. R. et al., (2005) Vitellogenin expression in queen ovaries and in larvae of both sexes of *Apis mellifera*. *Archives of Insect Biochemistry and Physiology*, 59, 211–218.
- Hartfelder, K. (2000) Insect juvenile hormone: from "status quo" to high society. *Brazilian Journal of Medical and Biological Research* 33(2), 157-177.

- Havukainen, H., et al., (2011) Social pleiotropy and the molecular evolution of honey bee vitellogenin. *Molecular Ecology* 20 (24), 5111-5113.
- Havukainen, H., et al., (2013) Vitellogenin recognizes cell damage through membrane binding and shields living cells from reactive oxygen species. *Journal of Biological Chemistry*, 288, 28369-28381.
- Hayward, A, et al. (2010) Comparative genomic and phylogenetic analysis of vitellogenin and other large lipid transfer proteins in metazoans. *FEBS letters*, 584 (6), 1273-1278.
- He, X. J., et al. (2017) Making a queen: an epigenetic analysis of the robustness of the honeybee (*Apis mellifera*) queen developmental pathway. *Molecular Ecology*, 26 (6), 1598-1607.
- Ihle, K. E., et al. (2010) Genotype effect on regulation of behaviour by vitellogenin supports reproductive origin of honeybee foraging bias. *Animal behaviour* 79 (5), 1001-1006.
- Jackson, J, T., et al., (2011) Histological estimates of ovariole number in honey bee queens, *Apis mellifera*, reveal lack of correlation with other queen quality measures. *Journal of Insect Science*, 11 (1), 82.
- Jedlička P., et al., (2016) Gene expression dynamics in major endocrine regulatory pathways along the transition from solitary to social life in a bumblebee, *Bombus terrestris*. *Frontiers in Physiology*, 7:574.
- Kapheim, K. M., Johnson, M. M. (2017) Juvenile hormone, but not nutrition or social cues, affects reproductive maturation in solitary alkali bees (*Nomia melanderi*). *Journal of Experimental Biology*, 220.20, 3794-3801.
- Kent, C. F., et al. (2011) Adaptive evolution of a key gene affecting queen and worker traits in the honey bee, *Apis mellifera*. *Molecular Ecology* 20 (24), 5226-5235.
- Kocher, S. D., et al., (2008) Genomic analysis of post-mating changes in the honey bee queen (*Apis mellifera*). *BMC Genomics*, 232.
- Kucharski, R. et al. (2008) Nutritional control of reproductive status in honeybees via DNA methylation. *Science*, 319, 1827–1830.
- Li, A. K., et al., (2003) Receptor-ligand interaction between vitellogenin receptor (VtgR) and vitellogenin (Vtg), implications on low density lipoprotein receptor and apolipoprotein B/E - The first three ligand-binding repeats

- of VtgR interact with the amino-terminal region on Vtg. *Journal of Biological Chemistry*, 278, 2799–2806.
- Li, Z. et al., (2008) Vitellogenin functions as a multivalent pattern recognition receptor with an opsonic activity. *PLoS One*, 3 (4).
- Maleszka, R., et al. (2014) Epigenomics and the concept of degeneracy in biological systems. *Briefings in Functional Genomics*, 13, 191-202.
- Mann, C. J., et al., (1999) The structure of vitellogenin provides a molecular model for the assembly and secretion of atherogenic lipoproteins. *Journal of Molecular Biology*, 285(1), 391-408.
- Murphy, C. A., et al. (2009) Testing and applying a fish vitellogenesis model to evaluate laboratory and field biomarkers of endocrine disruption in Atlantic croaker (*Micropogonias undulatus*) exposed to hypoxia. *Environmental Toxicology and Chemistry: An International Journal* 28 (6), 1288-1303.
- Naiem, E-S., et al., (1999) Nurse bees support the physiological development of young bees (*Apis mellifera* L.). *Journal of Comparative Physiology B* 169(5), 271-279.
- Nelson, C. M., et al. (2007) The gene vitellogenin has multiple coordinating effects on social organization. *PLoS Biol*, 5 (3).
- Nilsen, K., et al. (2011) Insulin-like peptide genes in honey bee fat body respond differently to manipulation of social behavioral physiology. *Journal of Experimental Biology*, 214 (9), 1488-1497.
- Peng, L., et al. (2020) CRISPR/Cas9-mediated vitellogenin receptor knockout leads to functional deficiency in the reproductive development of *Plutella xylostella*. *Frontiers in Physiology*, 10, 1585.
- Piulachs, M. D., et al., (2003) The vitellogenin of the honey bee, *Apis mellifera*: structural analysis of the cDNA and expression studies. *Insect Biochemistry and Molecular Biology*, 33 (4), 459-465.
- Raikhel, A. S., et al., (2005) Hormonal control of reproductive processes. *Comprehensive Molecular Insect Science*, 3, 433-491.
- Rangel, J. et al. DR (2012) The effects of honey bee (*Apis mellifera* L.) queen reproductive potential on colony growth. *Insectes Sociaux*, 60, 65–73.

- Roth, Z., et al. (2013) Identification of receptor-interacting regions of vitellogenin within evolutionarily conserved β -sheet structures by using a peptide Array. *Chembiochem*, 14 (9), 1116-1122.
- Rutz, W., Lüscher, M. (1974) The occurrence of vitellogenin in workers and queens of *Apis mellifica* and the possibility of its transmission to the queen. *Journal of Insect Physiology* 20 (5), 897-909.
- Saiki, R., et al., (2015) High juvenile hormone titre and abdominal activation of JH signalling may induce reproduction of termite neotenics. *Insect Molecular Biology*, 24, 432–441.
- Salmela, H. (2015) Transfer of immunity from mother to offspring is mediated via egg-yolk protein vitellogenin. *PLoS Pathogens*, 11 (7).
- Salmela, H., et al. (2016) Ancient duplications have led to functional divergence of vitellogenin-like genes potentially involved in inflammation and oxidative stress in honey bees. *Genome Biology and Evolution*, 8 (3), 495-506.
- Schultz, I. R., et al. (2001) Dose–response relationships and pharmacokinetics of vitellogenin in rainbow trout after intravascular administration of 17 α -ethynylestradiol. *Aquatic Toxicology*, 51 (3), 305-318.
- Seehuus, Siri-Christine, et al. (2006) Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *Proceedings of the National Academy of Sciences*, 103 (4), 962-967.
- Seehuus, Siri-Christine, et al. (2007) Immunogold localization of vitellogenin in the ovaries, hypopharyngeal glands and head fat bodies of honeybee workers, *Apis mellifera*. *Journal of Insect Science*, 7 (1), 52.
- Shen, Y., et al., (2019) Vitellogenin and Vitellogenin-Like genes in the Brown Planthopper. *Frontiers in Physiology*, 10, 1181.
- Shi, Y. Y. et al. (2011) Diet and cell size both affect queen-worker differentiation through DNA methylation in honey bees (*Apis mellifera*, Apidae). *PLoS One*, 6, e18808.
- Trenczek, T. et al., (1989) Developmental patterns of vitellogenin haemolymph titre and rate of synthesis in adult drone honey bees (*Apis mellifera*). *Journal of Insect Physiology* 35 (6), 475-481.

- Tufail, M., Takeda, M. (2009) Insect vitellogenin/lipophorin receptors: Molecular structures, role in oogenesis, and regulatory mechanisms. *Journal of Insect Physiology*, 55 (2), 88-104.
- Wang, R., et al. (2010) Cloning, expression, and induction by 17- β estradiol (E 2) of a vitellogenin gene in the white cloud mountain minnow *Tanichthys albonubes*. *Fish Physiology and Biochemistry* 36 (2), 157-164.
- Wheeler, D. E., Kawooya, J. K. (1990) Purification and characterization of honey bee vitellogenin. *Archives Of Insect Biochemistry And Physiology*, 14 (4), 253-267.
- Wheeler, M. M., et al. (2013) Brain gene expression changes elicited by peripheral vitellogenin knockdown in the honey bee. *Insect Molecular Biology* 22 (5), 562-573.
- Winston, M. L. (1987) *The Biology of the Honey Bee*. Cambridge, Massachusetts, Harvard University Press.
- Woodard, S. H. et al. (2011) Genes involved in convergent evolution of eusociality in bees. *Proceeding of the National Academy of Science of the United States of America*, 108, 7472–7477.
- Woyke, J. (1971) Correlations between the age at which honeybee brood was grafted, characteristics of the resultant queens, and results of insemination. *Journal of Apicultural Research*, 10, 45–55.
- Wurm, Y., et al. (2011) The genome of the fire ant *Solenopsis invicta*. *PNAS*, 108, 5679-5684.
- Zhang, H., et al. (2019) Identification and characterization of the vitellogenin receptor gene and its role in reproduction in the Alligatorweed Flea Beetle, *Agasicles hygrophila*. *Frontiers in Physiology*, 10, 969.
- Zhen, C., et al. (2018) Broad-complex Z3 contributes to the ecdysone-mediated transcriptional regulation of the vitellogenin gene in *Bombus lantschouensis*. *PLoS One*, 13 (11).

CHAPTER III

A rapid and inexpensive histological procedure for reproduction associated-trait in *Apis mellifera* virgin queens

O artigo a seguir está redigido de acordo com as normas para publicação na revista *Apidologie* como **Short-Communication**.

CHAPTER III - A rapid and inexpensive histological procedure for reproduction associated-trait in *Apis mellifera* virgin queens

The female reproductive system of insects, including hymenopterans, is commonly formed by two ovaries composed by elongated tubular ovarioles which contain the oocytes (Büning, 1994). Generally, Apidae members have four or more ovarioles per ovary but honeybee queens, *Apis mellifera* L. (Hymenoptera: Apidae), have more than one hundred ovarioles per ovary, each typically consisting of 120–200 serial units (Snodgrass, 1956 and Linksvaer et al., 2011, as cited in Hartfelder et al., 2017). On the other hand, *A. mellifera* workers, which are non-reproductive bees, have fewer than ten ovarioles per ovary (Chaud-Netto and Bueno, 1979). This difference in the number of ovarioles among workers and queens is an outcome of programmed cell death of the ovarian tissue during the fifth larval instar of workers occurred by the lack of feeding royal jelly (Reginato and Cruz-Landim, 2003).

The honeybee queen is responsible for colony formation, being the mother of all individuals in a hive. The reproductive potential, physical traits, reproductive performance, lifespan and genetic variability of the offspring of a honeybee queen depend upon the queen phenotype as well as the number and genetic diversity of drones involved in mating (Camazine et al. 1998; Delaney et al. 2011; Moore et al., 2015).

In commercial hives used for the production of bee products such as honey, royal jelly, propolis, and wax, as well as for pollination of commercial crops, the selection of adequate honeybee queens and drones is desirable to form colonies with high economic performance (Cobey et al., 2009). Queen attributes such as the number of ovarioles may predict the reproductive

potential of a queen and, consequently, brood size and pattern (Delaney et al., 2011). Besides, information on queens and drones phenotypes is essential to establish a successful breeding program.

Herein, we document the pace of a rapid and straightforward histological method to facilitate ovariole counting and as this is a destructive method, results obtained by the use of this technique should be correlated with the observation of other non-destructive attributes of virgin queens, i.e. physical, morphological or molecular, to guide the selection of the most promising queens for setting up commercial hives.

Histological procedures were performed in the Department of Veterinarian Pathology at Sao Paulo State University, Jaboticabal, Brazil.

Histological Procedures

Fixation, Dehydration, Diaphanization and Infiltration

Queens were anaesthetized using CO₂, decapitated and pinned onto a dissection plate for ovary dissection under a stereomicroscope. Ovaries were carefully removed with forceps and immediately fixed in alcoholic Bouin's fixative solution (150 ml 80% ethanol, 60 ml 37% formaldehyde solution, 15 ml glacial acetic acid, and 1 g of a saturated picric acid solution - Presnell and Schreibman, 1997). After 4 hours fixation at room temperature, ovaries were transferred to 70% ethanol and kept overnight until complete dehydration. Fixated ovaries, were carefully transferred to a histological cage immersed in 90% ethanol for 10 min, followed by six immersions of 5 min each in 100% ethanol to complete dehydration. Then, samples were absorbed into a 1:1

solution of 100% ethanol: xylene and then immersed in 100% xylene as following: *i*) 15 min of immersion and *ii*) 45 min of immersion.

Paraffin was melted at 60°C in an oven and samples inside histological cages were let for Paraffin infiltration for up to 3 h to prevent excess drying of tissues. At this point, freshly infiltrated tissues were cooled at room temperature and then re-melted prior embedding in 7 x 7 x 5 mm metal base moulds. The ovaries could be easily manipulated during this process because they were bright yellow-coloured by the picric acid of the Bouin solution. Ovaries and testes were carefully oriented in the base moulds using heated forceps under a stereomicroscope to produce a consistent plane of section. Then, ovaries were placed in the corner of the mould for support and the lateral oviduct placed touching the base of the mould, so that it would be the first structure sectioned. Ovaries and testes were placed in the proper orientation using heated forceps grasping the distal tip of the ovaries as Paraffin cooled. It was always possible to return a poorly oriented embedded ovary and testes by re-melting Paraffin at 60°C in an oven, adjusting for the correct position and re-embedding. Before section, the blocks were place in a freezer (-4°C) for 20 min.

Paraffin blocks containing the embedded ovaries and testes were sectioned using a rotary microtome with heavy-duty high profile disposable microtome blades. Slices of 3-7 µm-thick were placed in slide inside a water tank and, when slices were visibly flattened, the water was drained from the slide. Later sections containing oocytes with less yolk and smaller trophocytes are easier to count (Jackson et al., 2011) and these sections are preferred for mounting and staining. The paraffin in the sections at this point, were melted in a oven at 60 °C for 1 h.

Paraffin removal, Rehydration, Staining and Dehydration

Immediately prior to staining, sections were immersed in xylene (two changes, 25 minutes each) for Paraffin removal and washed three times of 5 seconds each in 100% ethanol. Samples were immersed in an alcoholic solution of lithium carbonate (0.25%) for 30 minutes to remove the yellow picric acid from tissues (Presnell and Schreibman, 1997). After 1 minute dipped in distilled water, samples were stained for 2.5 minutes in filtered haematoxylin solution (50 ml of 10% alcoholic hematoxylin solution, 2.5 g of mercuric oxide, 20 ml of concentrated glacial acetic acid). Samples were stained in alcoholic eosin prepared by combining 50 ml of 1% eosin, 5 ml of 1% phloxine, 400 ml of alcohol 95% and 2 ml of glacial acetic acid. Three washes of 3 seconds each removed excess of stain in 100% ethanol. Samples were quickly immersed into a 1:1 solution of 100% ethanol: xylene, dipped promptly in 100% xylene two times following the third immersion in 100% xylene until mounting in microscopy slides. Then the slides were mounted with a coverslip and All-Purpose Varnish, Acrilex (xylene-based) – which is considerably cheap in comparison with Entellan or Canada Balsom. Slides were stored flat for 24 hours at room temperature (27°C), then to ensure adequate drying of the mounting before microscopy.

Images were captured in an Olympus BX50 microscope using an Olympus DP73 camera, and ovarioles were counted using the Cell counter tool of the software ImageJ version 1.52j. This method is faster in comparison with other protocols, e.g. Jackson et al. (2011), once it takes only four days to be completed since dissection. When compared in terms of logistical and reagent

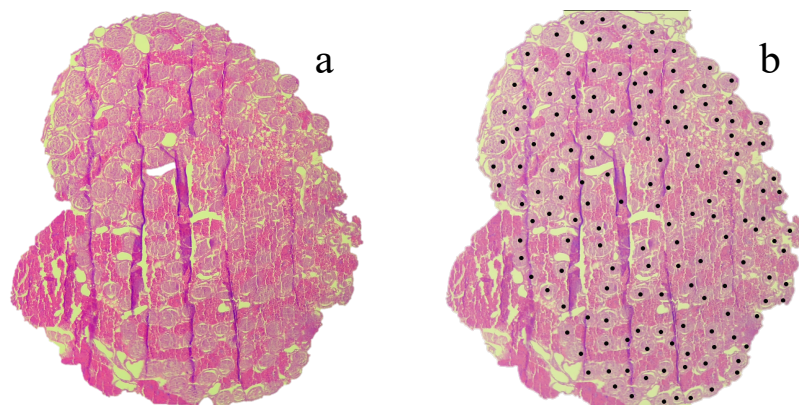
costs, the present protocol was also found to be cheaper than the one described by Jackson et al. (2011) and Raulino et al. (2019). (Table 1).

Table 1. Comparison time per protocol and estimated costs based on the proposed protocol and previous studies.

Authors	Approximated time per protocol	Estimated cost per thirty samples (USD)
Proposed protocol	14h (3 working days)	28
Jackson et al. (2011)	24h (4 working days)	35
Raulino et al. (2019)	10h (2 working days)	32

High-quality images were generated using the described protocol making ovariole counting easy (Figure 1, a and b).

Figure 1. (a) Example of *Apis mellifera* ovarioles of a virgin queen viewed in cross section (b) and the same section with marked ovarioles (n=149) by using a counting tool on ImageJ software.



References

- Büning, J. (1994) The insect ovary: ultrastructure, previtellogenic growth and evolution. Springer Science & Business Media.
- Camazine, S., et al., (1998) How healthy are commercially-produced U.S. honey bee queens? *American Bee Journal*, 138, 677-680.
- Chaud-Netto, J., Bueno, O. C. (1979) Number of ovarioles in workers of *Apis mellifera adansonii* and *Apis mellifera ligustica*: A comparative study. *Journal of Apicultural Research*, 18: 260-263.
- Cobey, S., et al., (2009) Status of breeding practices and genetic diversity in domestic US honey bees. *Honey bee colony health: Challenges and Sustainable Solutions*. CRC, Boca Raton, Florida, 39-49.
- Delaney, D. et al., (2011) The physical, insemination, and reproductive quality of honey bee queens (*Apis mellifera* L.). *Apidologie*, 42 (1), 13.
- Hartfelder, K., et al., (2017) The ovary and its genes – developmental processes underlying the establishment and function of a highly divergent reproductive system in the female castes of the female castes of the honey bee, *Apis mellifera*. *Apidologie* 49, 70.
- Jackson, J. T., et al., (2011) Histological estimates of ovariole number in honey bee queens, *Apis mellifera*, reveal lack of correlation with other queen quality measures. *Journal of Insect Science*, 11, 82.
- Moore, P. A., et al., (2015) Honey Bee Queens: Evaluating the Most Important Colony Member. *Bee Health*, 7, 10.
- Presnell, J. K., Schreibman, M. P. (1997) *Humason's Animal Tissue Techniques*, 5th edition. Johns Hopkins University Press.

- Raulino-Domanski, F., et al. (2019) Optimized histological preparation of ovary for ovariole counting in Africanized honey bee queens (Hymenoptera: Apidae). *Journal of Insect Science* 19 (2), 12.
- Reginato, R. D., Cruz-Landim, C. (2003) Ovarian growth during larval development of queen and worker of *Apis mellifera* (Hymenoptera: Apidae). *Brazilian Journal of Biology*, 63, 121-127.
- Sarikaya, D. P., et al., (2012) The roles of cell size and cell number in determining ovariole number in *Drosophila*. *Developmental Biology*, 363, 279–289.

CHAPTER IV

Association between *Vg* gene polymorphisms and weight at emergence in *Apis mellifera* queens

O artigo a seguir está redigido de acordo com as normas para publicação na revista *Apidologie*, onde foi submetido em setembro de 2020.

CHAPTER IV - Association between *Vg* gene polymorphisms and weight at emergence in *Apis mellifera* queens

Abstract

Vitellogenin gene (*Vg*) has been pointed out as a key factor affecting hormone signaling related to ovary development and has been suggested to affect reproductive traits. Hence, the aim of this study was to identify *Vg* gene polymorphisms and their effect on weight at emergence in honeybees. The weight at emergence (WE) was measured for 238 honeybee queens from those, 25 queens were selected for high (H) and low (L) estimated breeding value (EBV). A total of 126 polymorphisms were identified, from those, four SNP markers showed a significant effect between H and L EBV groups. Among the most significant variants in *Vg* gene, LG4:5030917:C:T and LG4:5030919:C:G, were identified on exon 5, and linkage disequilibrium ($r^2 = 0.33$) indicated a moderate association among them. As these SNPs have an effect on the protein translation, this can explain the change in weight at emergence by a possible effect on the ovary development - the major factor related to affect weight at emergence.

Keywords: Honeybee, PCR-sequencing, vitellogenin, SNP.

Introduction

Honeybee queen selection has great importance to enhance the brood and honey production at colony level (Rangel et al., 2013). Recently, the evaluation of the queens for the reproductive and morphological aspects, have been gradually become target traits for honeybee breeding aiming to improve productivity of the hive (Akyol et al. 2008). Regarding this aspect, the vitellogenin gene (*Vg*) has been extensively studied in honeybees due their pleiotropic effect in honeybees and workers.

Identification, accumulation and perpetuation of favorable genes for quantitative traits may be facilitated if the selection is based on variance components and genetic parameters and it is only possible through quantitative genetics that is the key for genetic breeding and selection. In this way, genetic parameters allow us to identify the action nature of involved genes, as well as evaluating the efficiency of different selection methods and strategies, whether from cross-breeding or selection (Cobey et al., 2012).

Studying phenotypic traits makes possible to estimate genetic parameters from genotypic components, while the magnitude of parameter estimates defines the genetic patterns of the population. Thus, in possession of these data, the breeder has more information for decision-making, increasing selection efficiency. Besides, the understanding of the molecular basis associated with phenotypic variability in queens is a major outstanding question in honeybee's biology and beekeeping (Ronai et al. 2016). In modern techniques of selective breeding and systematics in beekeeping, the use of SNP markers has proved to be effective (Whitfield et al. 2006). SNP sites scattered over the honeybee genome have been used for subspecies

identification, estimation of the introgression rate, and bee breeding all over the world (Pinto et al. 2014).

As a major precursor of honeybee yolk protein, vitellogenin is also known to be a high-density (Mw of 180 kDa) monomeric phospholipid glycoprotein (Tufail and Takeda, 2008). It was found previously that *Vg* has a pleiotropic effect, leading to the appearance of various phenotypic traits in queens and worker honeybees (Amdam et al., 2003). The concentration of vitellogenin in queens was proved to correlate positively with the egg production rate (Excels, 1974) and to play an important role in the caste differentiation process (Seehuus et al., 2006). Delaney et al. (2011) reported that the levels of vitellogenin transcription may be associated with the queens' weight and morphometrics.

In honeybees, vitellogenin is produced by fat bodies, released into the hemolymph, and cumulates in oocytes as a nutritional supply for the embryo (Tufail and Takeda, 2008). The honeybee *Vg* gene consists of seven exons and 6175bp; which are published in the GenBank database. The purpose of this study, based on comparative analysis of the honeybee *Vg* gene, was to find new, previously unknown SNPs that can be used in beekeeping as genetic markers, once they were proved to be related to emergence weight and further with reproductive and colony performance traits.

Material and Methods

Animals

The fieldwork was carried out at the Bee Research Lab facilities at São Paulo State University, Jaboticabal, Brazil. Twelve non-related colonies were evaluated and their health status (visually) and colony strength (number of

brood >6 and storage frames > 4 in the hive) were assessed. From those, a total of 238 queens were reared, by grafting larvae with specific age into plastic cup cells that were affixed to bar and then introduced into previously prepared hives, accordingly with Doolittle method (Doolittle, 1915). Queen cells, were then taken to an incubator, ten days after the grafting, with controlled humidity (60-64%) and temperature (30-32°C). Emergence was permanently controlled and queens were weighted at emergence (N=238). From those, 50 honeybee queens were selected based on its high and low EBV (for emergence weight, for DNA extraction and sequencing).

Genotyping and sequencing

Procedures were in accordance with standard practices for the ethical handling of invertebrate samples (Sherwin et al., 2003), because *A. mellifera* is not a regulated invertebrate, no ethical use or institutional review board approval was required. DNA was extracted from the tissue of honeybee pectoral flight muscles using the Wizard® Genomic DNA Purification Kit (Promega, USA), some adjustments were made to the manufacturer's instructions (see supplementary material: Isolating Genomic DNA from Animal Tissue). *Vg* gene was amplified using PCR (see supplementary material: Table 1 for a list of primers).

PCR was performed on a T100 Thermal Cycler (Bio-Rad) using 25 µl reactions consisting of 10x High Fidelity PCR buffer, 5 U/µl Platinum Taq Polymerase High Fidelity, 50 mM MgSO₄, 10 mM dNTP mix, 10 µM primers and 20-100 ng of extracted DNA and MilliQ water. The PCR was carried out using a touchdown protocol with the thermal profile of 5 minutes at 95 °C, followed by 35 cycles of 95°C (35s), 58°C (30s) and 72°C (180s) and a final

extension at 72 °C for 5 minutes. No template controls were performed with each PCR run. Products were visualized by electrophoresis on 1.0% agarose gels and were purified with a Quick PCR Purification Kit (Promega, USA) for sequencing. Sequencing was performed by the Technological Research Center at Sao Paulo State University. The sequencing of PCR products was done using both primers (forward and reverse) and it was performed in an automated ABI 3130 sequencer (Applied Biosystems) using the kit BigDye Terminator v3.1 (Applied Biosystems).

Estimates of genetic parameters for weight at emergence

Estimates of variance components were obtained by using the BLUPF90 software (Misztal et al., 2015). A single-trait best linear unbiased prediction (BLUP) model was used to infer the genetic parameters for the WE according to the following model:

$$y = Xb + Zu + e$$

where y is the vector of phenotypic information for the WE, b is the random effect of the hives and u is the random effect of animals and e is the residual effect. The X and Z are the incidence matrices relating to effects b and u , respectively.

The random effects of the hive, animal and residual were assumed to be normally distributed: $b \sim (0, \mathbf{I} \otimes \sigma_b^2)$, $a \sim (0, \mathbf{A} \otimes \sigma_a^2)$ and $e \sim (0, \mathbf{I} \otimes \sigma_e^2)$, in which the σ_b^2 , σ_a^2 and σ_e^2 are the hive, additive genetic and residual variances components, \mathbf{A} represents the relationship matrix based on pedigree information, \mathbf{I} is an identity matrix and \otimes is the Kronecker product.

Verification of SNPs

The quality of obtained sequences of the *Vg* gene were analyzed by the program FASTQC (Andrews et al., 2010). Base calling were performed by the program Tracy (Rausch et al, 2020) and bases with quality score in Phred Scale lower than 20 were discarded, as well as sequences with length lower than 100 bases.

Sequences were aligned using BWA (Li and Durbin, 2009) with reference to the *Vg* gene sequences of *A. m. mellifera* published in NCBI (version Amel_HAV3.1, GCF_003254395.2; NC_037641.1, position range from 5029485 to 5035661, of the fourth chromosome LG4 of the honey bee. Variant calling were performed using SAMtools program (Li, 2011) and quality control applied with VCFtools (Danecek et al., 2011).

The linkage disequilibrium (r^2) was estimated using the PLINK 1.9 (Purcell et al, 2007) program (available at <http://zzz.bwh.harvard.edu/plink/>) to determine which SNPs were more frequently inherited together. Considering two loci with two alleles for each locus (A1/A2 and B1/B2), the following formula was used:

$$r^2 = D^2/[f(A1)*f(A2)*f(B1)*f(B2)] \text{ (Hill and Robertson, 1966),}$$

where $D = f(A1_B1)*f(A2_B2) - f(A1_B2)*f(A2_B1)$ (Hill, 1981).

The program compares the observed and expected frequencies of the SNPs in order to see if they are in linkage disequilibrium or not. If they are in linkage disequilibrium, they may have the same statistical association with the trait.

The following statistical model, Cochran-Armitage Trend Test (Armitage, 1955), was applied to evaluate the associations between SNPs and the phenotypic data, based in two groups (for high and low EBV) of the trait studied:

$$Z_T = 2\sqrt{rs}(p_{cases} - p_{controls})/\sqrt{4n_2 + n_1 - 4np^2}$$

To test $H_0: p_{cases} = p_{controls}$, without assuming Hardy-Weinberg Equilibrium. Where Z_T is approximately $N(0,1)$ under H_0 . Z_T^2 is approximately χ^2 with one degree of freedom. Where, p corresponds to the allelic frequency and n to the number of individuals.

The association level was estimated for the weight at emergence trait, the allelic substitution effects was calculated using the Variant Effect Predictor (Mclaren et al., 2016) from *Ensembl* and genomic annotation from AmelHAV3.1 (GCF_003254395.2), available on NCBI. Considering the association test, the allelic substitution effect a dominant/recessive approach was taken into account, and was considered 1 for the reference homozygous or heterozygous allele and 0 for the variant homozygous.

The number of animals used for statistical analysis was 50. Data manipulation, plots and other statistical analysis were performed using the software RStudio for R language (TEAM, R., 2013; RStudio TEAM, et al, 2015).

Results

Two groups of animals were formed based on estimated breeding value (EBV) for WE, in low (L) (-27.61 ± 9.85) and high (H) (38.55 ± 11.84). We tested EBV groups using T-student Test, which were significantly different ($p.value < 3.2e^{-35}$). A total of 50 animals were selected, 25 animals from the

lower and 25 from the group with higher values. The phenotypic mean for weight at emergence was $199.43 \pm 25.46\text{mg}$ (N=238). Fifty *Vg* gene sequences were obtained for honeybees and we assessed to RefSeq database for *Vg* reference gene under the following accession number: NC_037641.1. The genomic annotation information was obtained from NCBI by GCF_00325495.1 accession number and for the *Vg* gene region in NM_001011578.1. Hundred and twenty-six polymorphisms were identified in the fragments of the *Vg* gene, including intronic and exonic regions, amplified from 50 queens. Four of those were significant SNPs (Figure 1).

The SNPs had a phred quality bigger than 20, it means an error probability of 0.01. The SNPs were named accordingly to the position in the DNA sequences deposited in GenBank. One SNP is located in exon 3 (5033692:C:T), two SNPs are located in exon 5, position 5030917 (C:T) and 5030919 (C:G). The fourth was found in exon 6, position 5030215 (C:A). All SNPs were found to be in Hardy-Weinberg equilibrium ($p\text{-value} < 0.0001$). Both of the SNPs located in exon 5 cause missense mutations, the location in the gene, type of substitution, amino acid change and Grantham score are in Table 2. Allele 5030917 (C:T), in particular, occurred considerably more in the Low (42%) than the High (12%) EBV group of animals. The frequency of the occurrence of the SNPs between groups (Low EBV and High EBV) are represented in Table 3. The linkage disequilibrium was estimated to determine if polymorphisms were segregated together. A r^2 higher than 0.33 was considered to indicate that SNPs were in strong linkage disequilibrium and were inherited together (Ardlie et al. 2002). In the present study, r^2 estimates ranged from 0.03 to 0.33 (Figure 2).

Discussion and Conclusion

Most of the significant SNPs presented r^2 values <0.33 between one another, indicating that they are mostly inherited separately. However, the higher r^2 value (0.33) was found between SNPs 5030917 (C:T) and 5030919 (C:G), demonstrating that these two are more frequently inherited together, which was expected given their proximity within the gene. We also analyzed intronic region but none of the SNPs were significant, and so it is expected that these SNPs are not in linkage disequilibrium with some other polymorphism, or that they affect some microRNA production site (Le Hir et al. 2003). Which means that they may not be interfering with the transcription of other genes. Considering SNP 5030917 (C:T) causes change in the protein translation by substituting a Glycine by an Aspartic acid, albeit a moderately conservative change considering the Grantham score (94), this may lead to morphometric and reproductive differences between animals.

These results provide a genetic basis for further studies in molecular and morphological mechanisms. Studies investigating genes expressed in the tissues of queens reproductive system and polymorphisms in other genes should be conducted to identify causal mutations that explain reproductive fitness. This is the first study showing a significant association between polymorphisms in the *Vg* gene associated with queens' weight at emergence.

Although, Ilyasov et al. (2015) identified the *Vg* gene as a possible candidate to act as a genetic passport to identified branches, by finding 26 SNP sites that differentiate two *Apis mellifera* evolutionary branches (M and C). The study of genes expressed in the tissues of ovaries or other tissues associated

with the reproductive system are important for a better understanding of the dynamics of weight at emergence and its implications in honeybees. With this in mind, future studies investigating polymorphisms in other genes acting on the ovaries and reproductive system may help to unravel markers for animal genetic evaluation. This study characterized the intronic and exonic regions of the *Vg* gene in Africanized honey bees. To date, this is the first study investigating the *Vg* gene has being affecting the weight at emergence of virgin queen.

Polymorphisms were detected in the studied regions, indicating the variability of the loci analyzed. Two of the SNPs identified cause amino acid substitutions and would be candidates for association studies considering reproductive traits related to weight at emergence. The association between *Vg* gene polymorphisms and weight at emergence demonstrates the influence of this gene can have on morphometrics in honeybees. The study of this gene expressed in tissues, especially in ovaries of queens selected by its genetic value of weight at emergence, is necessary since their influence on reproductive performance and dynamics has been little explored so far.

References

- Akyol, E. et al (2008) An observation study on the effects of queen age on some characteristics of honey bee colonies. *Ital. J. Anim.* 7(1), 19-25.
- Amdam, G. V. et al (2003) Social exploitation of vitellogenin. *PNAS* 100(4), 1799-1802.
- Andrews, S. et al (2010) FastQC: a quality control tool for high throughput sequence data. Babraham Bioinformatics.

- Ardlie, K. G. et al (2002). Patterns of linkage disequilibrium in the human genome. *Nat. Rev. Genet.* 3(4), 299-309.
- Armitage, P. (1955) "Tests for linear trends in proportions and frequencies." *Biometrics* 11.3: 375-386.
- Chen, J. S. et al (1997) Extensive sequence conservation among insect, nematode, and vertebrate vitellogenins reveals ancient common ancestry. *J. Mol. Evol.* 44, 440–451.
- Cobey, S. et al (2012) Status of breeding practices and genetic diversity in domestic US honey bees. *Honey bee colony health: Challenges and Sustainable Solutions*. CRC, Boca Raton, Florida, 39-49.
- Cridland, J. M. et al (2017) The complex demographic history and evolutionary origin of the western honey bee, *Apis mellifera*. *Genome Biol. Evol.* 9(2), 457-472.
- Danecek, P. et al (2011) The variant call format and VCFtools. *Bioinformatics*, v. 27, n. 15, p. 2156-2158, 2011.
- Delaney, D. et al. (2011) The physical, insemination, and reproductive quality of honey bee queens (*Apis mellifera* L.). *Apidologie*, 42 (1), 13.
- Doolittle, G. M. (1915) Scientific queen-rearing as practically applied: being a method by which the best of queen-bees are reared in perfect accord with nature's ways. *American Bee Jr.*
- Excels, W. (1974) Occurrence and significance of vitellogenins in female castes of social Hymenoptera. *Am. Zool.* 14(4), 1229-1237.
- Hill, W. G., Robertson, A. (1966) The effect of linkage on limits to artificial selection. *Genet. Res.* 8(3), 269-294.

- Hill, W. G. (1981) Estimation of effective population size from data on linkage disequilibrium. *Genet. Res.* 38(3), 209-216.
- Ilyasov, R. A. et al (2015) New SNP markers of the honeybee vitellogenin gene (Vg) used for identification of subspecies *Apis mellifera mellifera* L. *Russ. J. Genet.* 51(2), 163-168.
- Kent, C. F. et al (2011) Adaptive evolution of a key gene affecting queen and worker traits in the honey bee, *Apis mellifera*. *Mol. Ecol.* 20(24), 5226-5235.
- Le Hir, H. et al (2003) How introns influence and enhance eukaryotic gene expression. *Trends in Biochem. Sci.* 28(4), 215-220.
- Li, H. (2011) A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. *J. Bioinform.* 27(21), 2987-2993.
- Li, H., Durbin, R. (2009) Fast and accurate short read alignment with Burrows–Wheeler transform. *J. Bioinform.* 25(14), 1754-1760.
- Mclaren, W. et al (2016) The Ensembl variant effect predictor. *Genome Biol.* 17(1), 122.
- Misztal, I. et al (2015) Manual for BLUPF90 family of programs. University of Georgia, Athens, USA.
- Pinto, M. A. et al (2014) Genetic integrity of the dark European honey bee (*Apis mellifera mellifera*) from protected populations: a genome wide assessment using SNPs and mtDNA sequence data. *J. Apic. Res.* 53(2), 269–278.
- Purcell, S. et al (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum Genet.* 81(3), 559-575.

- Rangel, J. et al (2013) The effects of honey bee (*Apis mellifera* L.) queen reproductive potential on colony growth. *Insectes Soc.* 60(1), 65-73.
- Rausch, T. et al (2020) Tracy: basecalling, alignment, assembly and deconvolution of sanger chromatogram trace files. *BMC Genomics*, 21(1), 1-9.
- Ronai, I. et al (2016) The mechanistic, genetic, and evolutionary basis of worker sterility in the social Hymenoptera. *Adv. Study Behav.* 48, 251–317.
- Sappington, T. W., Raikhel, A. S. (1998) Molecular Characteristics of insect vitellogenin and vitellogenin receptors. *Insect Biochem. Mol. Biol.* 28, 277– 300.
- Seehuus, S. C. et al (2006) Reproductive protein protects functionally sterile honey bee workers from oxidative stress. *PNAS* 103(4), 962-967.
- Sherwin, C. M. et al (2003) Guidelines for the ethical use of animals in applied ethology studies. *Appl. Anim. Behav. Sci.* 81(3), 291-305.
- Team, R. Core. R development core team (2013) *RA Lang Environ Stat Comput*, 55, 275-286.
- Team, RStudio et al. (2015) RStudio: integrated development for R. RStudio, Inc., Boston, MA URL <http://www.rstudio.com>, 42, 14.
- Tufail, M., Takeda, M. (2008) Molecular characteristics of insect vitellogenins. *J. Insect Physiol* 54(12), 1447-1458.
- Whitfield, C. W. et al (2006) Thrice out of Africa: ancient and recent expansions of the honey bee, *Apis mellifera*. *Science*, 314, 642–645.

Tables and Figures

Table 2. SNPs frequencies occurrence for the Low and High EBV groups.

Polymorphism	EBV Groups	
	L	H
5030215(C:A)	0	0.22
5030917(C:T)	0.42	0.12
5030919(C:G)	0.26	0.02
5033692(C:T)	0	0.1875

Table 3. Position, gene region, nitrogen-base substitution, amino acid substitution, NCBI accession number and the Grantham score of the polymorphisms identified.

*position based on the NC_037641.1 sequence deposited in GenBank

Polymorphism*	Primer pair	Region	Type of codon substitution	Amino acid change	Grantham score
5030215(C:A)	9	Exon 6	acG-acT	-	-
5030917(C:T)	7	Exon 5	gGt – gAt	Glycine/Aspartic acid	94 (moderately conservative)
5030919(C:G)	7	Exon 5	caG-caC	Glutamic acid/Histidine	40 (conservative)
5033692(C:T)	3	Exon 3	caG-caA	-	-

Figure 1. Manhattan plot of *Vg* gene association for High and Low EBV groups for the weight of emergence of *Apis mellifera* queens. The horizontal blackline represents the significance threshold $-\log_{10}(p.value=0.05)$ for markers considering an FDR of 5%.

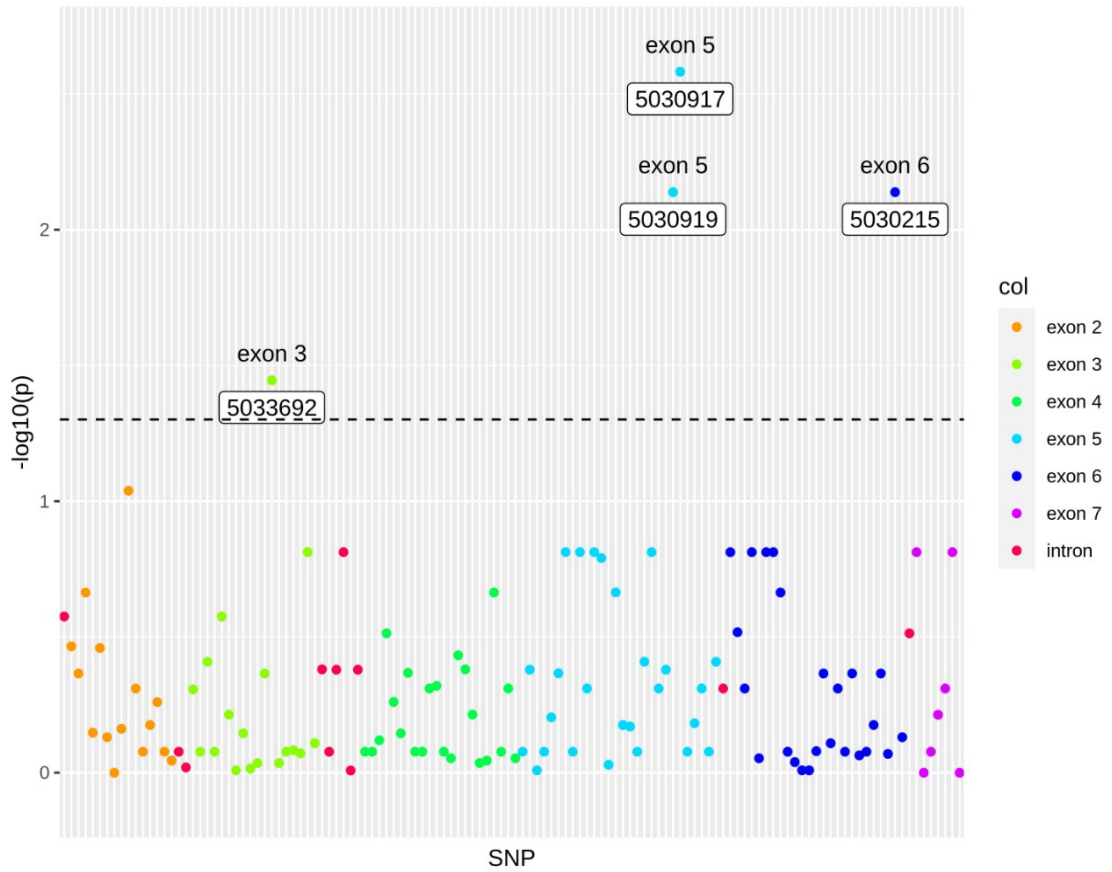
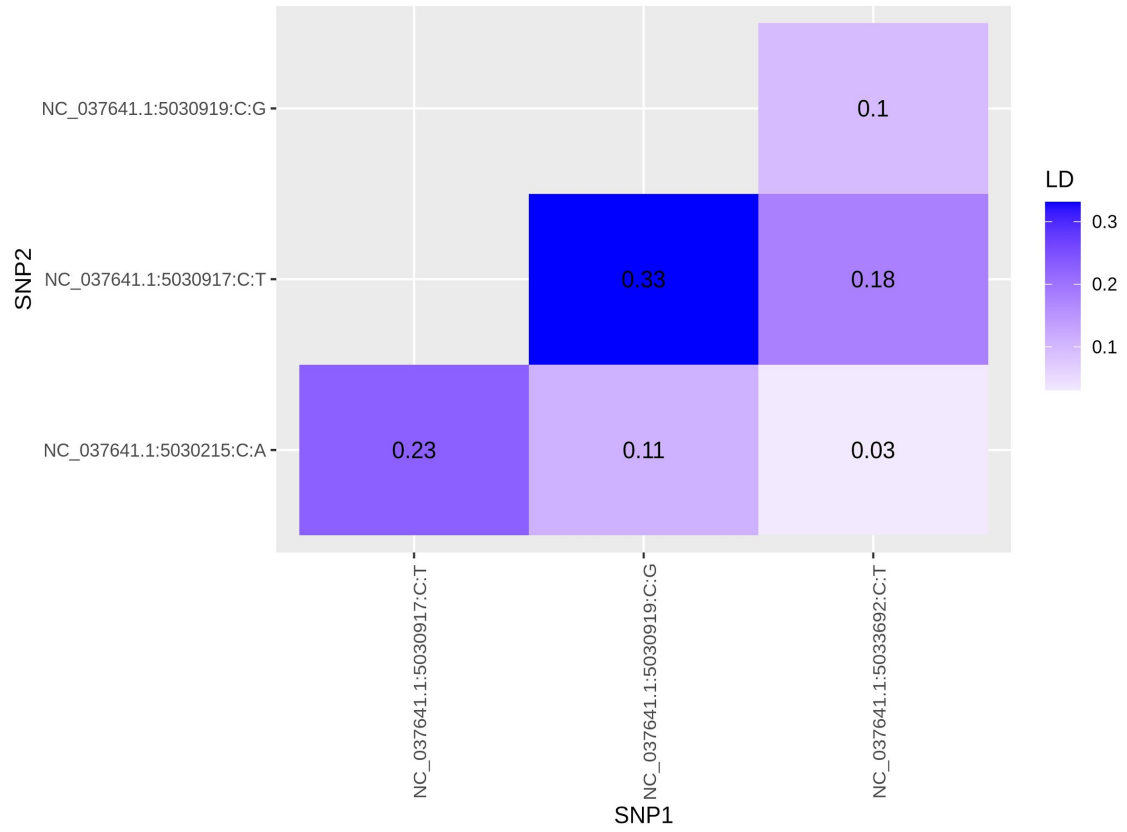


Figure 2. Linkage disequilibrium (LD), estimated pairwise r^2 values for the significant SNPs found on the *Vg* gene for weight at emergence trait.



Supplementary material

Isolating Genomic DNA from Animal Tissue

The following protocol was adapted from the Wizard® Genomic DNA Purification Kit (Promega, USA), for honeybee DNA extraction.

1. After grinding the thorax muscles, with nitrogen, allow the liquid to evaporate and transfer approximately 10-20mg of the ground tissue to 600µl of Nuclei Lysis Solution in a 1.5 ml microcentrifuge tube.
2. Incubate the lysate at 65°C for 30 minutes.
3. Add 17.5µl of 20mg/ml Proteinase K.
4. Add 3µl of RNase Solution to the nuclear lysate and mix the sample by inverting the tube 2-5 times. Incubate the mixture for 15-30 minutes at 37°C. Allow the sample to cool to room temperature for 5 minutes before proceeding.
5. To the room temperature sample, add 200µl of Protein Precipitation Solution and vortex vigorously at high speed for 20 seconds. Chill sample on ice for 5 minutes.
6. Centrifuge for 4 minutes at 12700rpm. The precipitated protein will form a tight white pellet.
7. Carefully remove the supernatant containing the DNA (leaving the protein pellet behind) and transfer it to a clean 1.5ml microcentrifuge tube containing 600µl of room temperature isopropanol. Some supernatant may remain in the original tube containing the protein pellet. Leave this residual liquid in the tube to avoid contaminating the DNA solution with the precipitated protein.

8. Gently mix the solution by inversion until the white thread-like strands of DNA form a visible mass.
9. Centrifuge for 2 min at 12700rpm at room temperature. The DNA will be visible as a small white pellet. Carefully decant the supernatant.
10. Add 600µl of 70% freeze ethanol and gently invert the tube several times to wash the DNA. Centrifuge 2 minutes at 12700rpm at room temperature.
11. Carefully aspirate the ethanol using a sequencing pipette tip. The DNA pellet is very loose at this point, and care must be used to avoid aspirating the pellet into the pipette.
12. Invert the tube on clean absorbent paper, and air-dry the pellet for 3 hours.
13. Add 100µl of DNA Rehydration Solution, and rehydrate the DNA by incubating at 65°C for 1 hour. Periodically mix the solution by gently tapping the tube. Alternatively, rehydrate the DNA by incubating the solution overnight at 4°C.
14. Store the DNA at 2-8°C.

Table 1. Primers design in order to cover *Vg* gene (introns and exons).

Primer	Sequence (5' – 3')		Amplicon size (bp)	Amplified region of <i>Vg</i>	Melting temperature
	Forward	Reverse			
1	ttctcgaaagttgtcttcaaca	agtcctccatggctttgaac	876	Beginning of the gene including intronic region to the middle of exon 2	51.5 °C
2	ctccgtgacctgcctatttc	ctcctcgacatccgagaaaag	862	Exon 2 to the beginning of exon 3	53.8 °C
3	gtcaaaacggattgggtgctt	tgttgaggaattgctcgttg	917	Exon 3 (partial) to exon 4 (partial)	49.7°C
4	tcaaggaatggatcgaaagg	tggttcacgctcctagcttt	828	End of exon 3 to exon 4 (partial)	51 °C
5	aaagctaggagcgtgaacca	gtgtcacgtccaggagat	865	Exon 4 (partial) to exon 5 (partial)	54 °C
6	gttcgagcatcgacacttca	aatcgccccttattctccac	717	Exon 5 (partial)	51.8 °C
7	gggagcagaaagatcctgtg	caaattgtcgatctcgctca	716	End of exon 5 to exon 6 (partial)	52 °C
8	ggggctacgtccataatcaa	cctggacaccacgattttct	782	Exon 6 (partial)	51.8 °C
9	cgctgcatgaccacgtatc	tgtcgtgatcacgtttctc	652	Exon 6 (partial) to exon 7 (partial)	51.8 °C
10	acgactcgaccaacgatttc	ttctggctgaggtcaggatt	454	Exon 7 (partial)	51.8 °C

Table 2. List of identified SNPs (N=126). Position in the gene, FDR and correspondent region (exon ID or intron). SNPs were listed per FDR value, from the lower to the higher.

Number	Polimorfism	FDR	Region
1	5030917:C:T	0.002619*	exon 5
2	5030919:C:G	0.007271*	exon 5
3	5030215:C:A	0.007271*	exon 6
4	5033692:C:T	0.03585*	exon 3
5	5034883:G:A	0.09152	exon 2
6	5030955:C:G	0.154	exon 5
7	5031577:A:T	0.154	exon 5
8	5030564:A:G	0.154	exon 6
9	5031663:G:A	0.154	exon 5
10	5033521:G:A	0.154	exon 3
11	5030475:T:A	0.154	exon 6
12	5031543:C:T	0.154	exon 5
13	5030524:G:A	0.154	exon 6
14	5033449:C:T	0.154	intron
15	5029845:A:G	0.154	exon 7
16	5030081:G:A	0.154	exon 7
17	5030450:G:A	0.154	exon 6
18	5031531:A:C	0.1619	exon 5
19	5031347:C:A	0.2166	exon 5
20	5030448:T:A	0.2169	exon 6
21	5032883:C:A	0.2169	exon 4
22	5035064:T:C	0.2169	exon 2
23	5035313:A:T	0.2658	intron
24	5034074:A:T	0.2658	exon 3
25	5030563:G:A	0.3037	exon 6
26	5033345:G:A	0.3066	exon 4
27	5030138:C:A	0.3066	intron
28	5035265:C:G	0.3423	exon 2
29	5034916:C:G	0.3472	exon 2
30	5032943:A:G	0.3693	exon 4
31	5034293:T:A	0.3901	exon 3
32	5030715:A:G	0.3901	exon 5
33	5031004:T:A	0.3901	exon 5
34	5032915:C:A	0.4162	exon 4
35	5033491:G:A	0.4162	intron
36	5030932:G:T	0.4174	exon 5
37	5031985:A:T	0.4174	exon 5
38	5033429:G:C	0.4174	intron
39	5033453:A:G	0.4174	intron
40	5033195:A:G	0.4287	exon 4
41	5031741:C:T	0.43	exon 5
42	5033698:C:T	0.4309	exon 3
43	5035258:A:C	0.4309	exon 2
44	5030317:A:T	0.4309	exon 6
45	5030370:T:A	0.4309	exon 6
46	5030398:C:G	0.4309	exon 6
47	5033014:T:C	0.4782	exon 4
48	5030545:T:C	0.4895	exon 6
49	5030796:G:C	0.4895	exon 5
50	5031565:A:C	0.4895	exon 5
51	5030382:G:A	0.4895	exon 6
52	5030644:aaagaa:aaa	0.4895	intron
53	5033105:C:T	0.4895	exon 4
54	5032872:G:A	0.4895	exon 4
55	5034858:T:C	0.4895	exon 2
56	5029920:A:C	0.4895	exon 7
57	5030941:G:T	0.4895	exon 5
58	5034379:G:A	0.4932	exon 3
59	5033339:G:C	0.5487	exon 4
60	5034806:G:T	0.5491	exon 2
61	5032911:T:A	0.6107	exon 4
62	5034055:G:A	0.6107	exon 3
63	5029946:G:A	0.6115	exon 7

64	5031958:G:A	0.6252	exon 5
65	5030820:C:T	0.6572	exon 5
66	5034831:A:T	0.6669	exon 2
67	5030337:G:A	0.6669	exon 6
68	5031322:T:C	0.6669	exon 5
69	5031096:A:T	0.6757	exon 5
70	5034884:T:C	0.6889	exon 2
71	5034938:A:C	0.7122	exon 2
72	5033220:C:T	0.716	exon 4
73	5033845:A:G	0.716	exon 3
74	5034890:A:T	0.7399	exon 2
75	5030179:A:C	0.7399	exon 6
76	5033375:G:A	0.7593	exon 4
77	5030390:T:A	0.7785	exon 6
78	5033506:G:A	0.7785	exon 3
79	5033548:C:T	0.8271	exon 3
80	5030399:T:A	0.8328	exon 6
81	5033162:G:T	0.8367	exon 4
82	5030380:T:G	0.8367	exon 6
83	5033158:T:C	0.8367	exon 4
84	5031025:T:C	0.8367	exon 5
85	5031657:A:G	0.8367	exon 5
86	5033465:T:C	0.8367	intron
87	5030341:G:T	0.8367	exon 6
88	5030442:T:A	0.8367	exon 6
89	5033389:A:C	0.8367	exon 4
90	5033378:G:A	0.8367	exon 4
91	5034509:C:T	0.8367	intron
92	5029961:A:G	0.8367	exon 7
93	5032878:T:A	0.8367	exon 4
94	5034802:A:T	0.8367	exon 2
95	5034282:C:A	0.8367	exon 3
96	5034313:G:A	0.8367	exon 3
97	5034854:G:A	0.8367	exon 2
98	5030789:T:C	0.8367	exon 5
99	5032010:A:T	0.8367	exon 5
100	5033001:T:A	0.8367	exon 4
101	5033593:C:T	0.8367	exon 3
102	5030906:A:C	0.8367	exon 5
103	5031969:G:T	0.8367	exon 5
104	5033533:T:C	0.8484	exon 3
105	5030310:T:C	0.8522	exon 6
106	5030350:C:A	0.8634	exon 6
107	5030501:G:A	0.8844	exon 6
108	5032988:G:T	0.8844	exon 4
109	5032867:T:A	0.8844	exon 4
110	5032890:T:C	0.9021	exon 4
111	5034741:A:T	0.9021	exon 2
112	5030414:T:A	0.9136	exon 6
113	5032910:T:G	0.921	exon 4
114	5033678:T:A	0.9215	exon 3
115	5033749:C:T	0.9215	exon 3
116	5031396:A:T	0.9346	exon 5
117	5034452:G:A	0.9564	intron
118	5033842:T:C	0.9651	exon 3
119	5033997:A:T	0.9795	exon 3
120	5031970:G:A	0.9795	exon 5
121	5030408:G:T	0.9795	exon 6
122	5030404:C:T	0.9795	exon 6
123	5033444:A:T	0.9808	intron
124	5029706:G:C	1	exon 7
125	5030030:A:G	1	exon 7
126	5034888:C:A	1	exon 2

*significant SNPs

CHAPTER V

Genetic passport of honey bees towards vitellogenin (Vg) SNP markers

O artigo a seguir foi redigido e contribuiu para a publicação do capítulo “The Origin of European Bees and their Intraspecific Biodiversity” do livro *Phylogenetics of Bees*, publicado em 2019 pela editora **CRC Press Taylor & Francis Group**.

CHAPTER V – Genetic passport of honey bees towards vitellogenin (*Vg*) SNP markers

Abstract

Assess the gene pool of honeybee subspecies is key to preserve the species as it is vital for beekeeping development worldwide. Mapping sites of single nucleotide polymorphism (SNP) has shown to be an effective method for genotyping honeybee colonies. The honeybee vitellogenin gene (*Vg*) encodes a protein that affects reproductive function, behavior, immunity, longevity, and social organization of the honeybee, while it is a conserved gene. The results of comparative analysis of honeybee *Vg* sequences show that there are 203 SNP sites that differentiate our samples (N=50), while considering the approach per evolutionary branches (M, A and C) and 204 SNPs in the contrast made per country. Our findings suggest that sampled animals from Jaboticabal, Brazil, are closer to the A lineage and from bees sampled from South Africa and Egypt. Here, based on our data, we highlight that *Vg* can be used as a possible hallmark of genetic passport to differentiate branches and to assess the proximity of evolutive lineages, when origin remains uncertain.

Honeybees are insects of great ecological and economic importance that, due to its role in pollination and honey production, and are now globally widespread. Its native range is large and diverse, and considerable variation can be observed within many populations and several can be further subdivided into a diversity of ecotypes. Ruttner (1988) developed and introduced the concept of evolutionary lineages (or branches) within *Apis mellifera*, by performing morphometric and genetic studies, in addition to analyses of ecophysiological, and behavioral traits. The taxonomy of honeybees remains

contradictory, while errors and issues still being found, much due to the lack of comprehensive and molecular evidence (Alburaki et al., 2011). Now there are five recognized, and genetically distinct, evolutionary branches distributed throughout Africa, Europe, and Asia: lineage A (African—*meda*, *adansonii*, *scutellata*, *monticola*, *litorea*, *capensis*, *unicolor*, *sahariensis*, *intermissa*, *ruttneri*, and the newly described *simensis*) (Meixner et al. 2014; Ilyasov et al. 2020); lineage C (subspecies east and south of the Alps including those along the northern Mediterranean, including *ligustica*, *carnica*, *macedonica*, *cecropia*, *cyprica*, and *adami*); lineage M (a west Mediterranean and northwest European—*mellifera* and *iberiensis*, but originally also including *intermissa*, *sahariensis*, *siciliana*, *ruttneri* (Sheppard et al. 1997), which were considered as links between the tropical African and the west Mediterranean subspecies); lineage O (Oriental—in the Near East and western Asia, including *caucasica*, *anatoliaca*, *syriaca*, *meda*, *armeniaca*, *jemenitica*, and the later described *pomonella* (Sheppard and Meixner 2003); and lineage Y (“*yemenitica*” from Ethiopia) (Sheppard and Meixner, 2003; Whitfield et al. 2006). Conventionally, intraspecific taxonomy of the honey bee has been based on morphology, and currently at least 33 subspecies of *A. mellifera* are known on the basis of morphometric traits (Ruttner 1988, Sheppard et al. 1997, Sheppard and Meixner 2003; Ilyasov et al. 2020).

Most recently, the increase in the availability of individual *Apis mellifera* genomes has resulted in significant progress towards a better understanding of its evolution (Kocher and Paxton 2014) and adaptation, therefore allowing for monitoring of the dispersal and admixture of honey bee populations. Furthermore, molecular studies have been crucial to reveal that several

subspecies and ecotypes can be considered as endangered (De la Rúa et al. 2009; Dogantzis and Zayed 2019), since several factors can lead to a loss of both genetic diversity and specific adaptations to local conditions (Meixner et al. 2013). In one of the first single nucleotide polymorphism (SNP) population genetics studies of *A. mellifera*, Whitfield et al. (2006) suggested that this species originated in Africa, colonized Europe and Asia, via two or three independent expansions. Chapman et al. (2016) developed an abbreviated SNP panel to assign the proportion of ancestry of honey bees to ancestral lineages of West European (M) and East European (C) based on 95 SNPs. During the last decades, morphometrical and molecular efforts have contributed to identifying new subspecies, evolutionary lineages, and a significant number of genes involved with adaptations and colony-level quantitative traits. Kent et al., (2011) using highly conserved group of genes (including *Vg*) concluded that *Vg* exhibits high levels of adaptive evolution in the genus *Apis*.

In Brazil, ~65 years ago, a small number of honeybees with African ancestry were introduced in Rio Claro, São Paulo State. This source population was derived from 47 queens of the subspecies *A. m. scutellata*, from South Africa and Tanzania (Kerr, 1957). Those bees, ended up dispersing, which led to a widely hybridization with existing managed populations of European origin, that quickly spread across much of the Americas. Besides, *Apis mellifera* are an invasive species in the Americas, introduced during the colonial times by the Europeans. Because of this event, Americas were firstly composed of M and C branches. The introduction of honeybees in South America, happened from the 1600s forward, probably mainly involving honeybees from the M evolutionary

branch (Kerr, 1957). The Africanization, how it is called this admixture event, is considered to be a spectacular example of a biological and rapid widespread invasion.

Vitellogenin belongs to a phylogenetically conserved group of egg yolk precursors and its genetic structure can contribute to explaining the adaptive evolution of honey bee. Vitellogenin as protein, leads to a double approach: it is central to egg production in the reproductive queen caste and a regulator of social behaviour in the sterile worker caste (Amdam et al. 2003). As an highly conserved gene, it has been under investigation not only as to have pleotropic effects on reproductive tasks but also as to be an indicator of branches differentiation and honeybee evolutionary pattern (Kent et al. 2011; Ilyasov et al. 2015). Here, we study case the genomic structure of the gene to assign the ancestry of the animals that were sampled. Aiming to unravel and underline the origin of the bees in our previously study regarding morphometric traits (see Chapter IV).

Vg sequences were sampled from 50 Africanized queens collected in Jaboticabal, São Paulo, Brazil. Comparative analysis of the *Vg* gene was performed accordingly with the reference sequences submitted to the GenBank database from different countries, representative of three different branches: A, M and C.

DNA was extracted from the tissue of honeybee pectoral flight muscles using the Wizard® Genomic DNA Purification Kit (Promega, USA) according to manufacturer's instructions. *Vg* gene was amplified using PCR (see supplementary material in Chapter IV: Table 1 for a list of primers).

The sequencing of PCR products was done using both primers (forward and reverse) and it was performed in an automated ABI 3130 sequencer (Applied Biosystems) using the kit BigDye Terminator v3.1 (Applied Biosystems).

The quality of obtained sequences in FASTQ format were concatenated and aligned with the genome reference sequence NC_037641.1, Amel_HAv3.1 using the BWA program. The nucleotide sequences (N=810) in the FASTA format of country and/or branch, available at NCBI, were used to identify variants among the sequenced samples contrasted with the evolutionary lineages A (N=142), C (N=192), M (N=156), samples (N=50) and *Apis cerana* (N=210), as the external species. Sequences of *Vg* for exons 2-7 were available for nine different countries (Croatia, Egypt, Germany, India, Poland, Russia, South Africa, Slovenia, Spain) (See Supplementary Material: Table 1 and Table 2).

Two different approaches to generate the cluster dendrogram, were carried out, one considering the available sequences per country (N=630), and the second, considering the sequences per branch (N=490). The latter, excluded India and Russia sequences from the dataset, since were no correspondent branches associated, for these countries. On the second approach, a threshold of >70% ancestry was adopted to assign a branch, each time a relative ancestry was available.

Sequences consensus (for branches, countries, external species and samples) were obtained by the BCFtools program. The variants were analyzed under RStudio program for R (pvclust package). The IBS matrix (identical by state) was estimated using the *dist* procedure with Euclidean Distance

Computational method by PLINK 1.9 (Purcell et al, 2007). The hierarchical clustering analysis was conducted using the *hclust* procedure with a complete method using the estimated IBS matrix. The dendrograms were generated by the *plot* procedure, approached by countries (Figure 1) and by evolutionary branches (Figure 2).

Figure 1. Cluster Dendrogram showing distance between sampled animals (“samples”) and with countries (Germany, Slovenia, Croatia, India, Egypt, South Africa, Spain, Poland, Russia), where *Vg* sequence data are available on GenBank. *Apis cerana* is representing an external species from the same genus. Values on the dendrogram are AU p-values (Red), and clusterlabels (grey, bottom). Clusters with AU >= 95% are indicated by the rectangles.

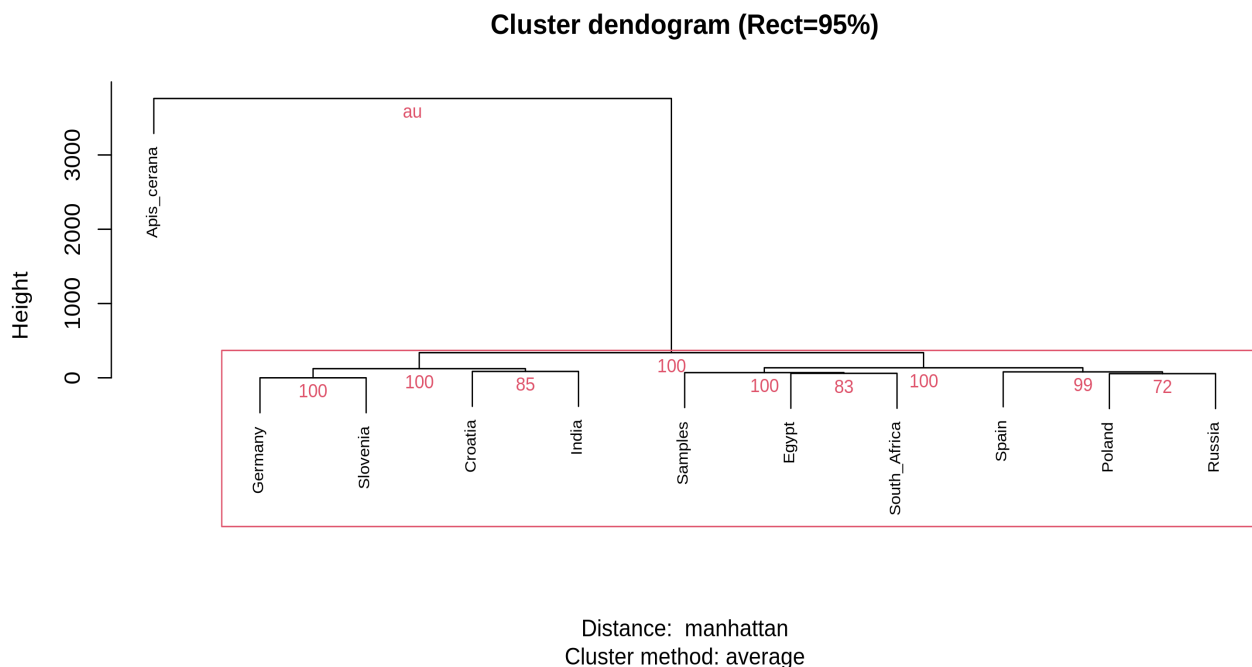
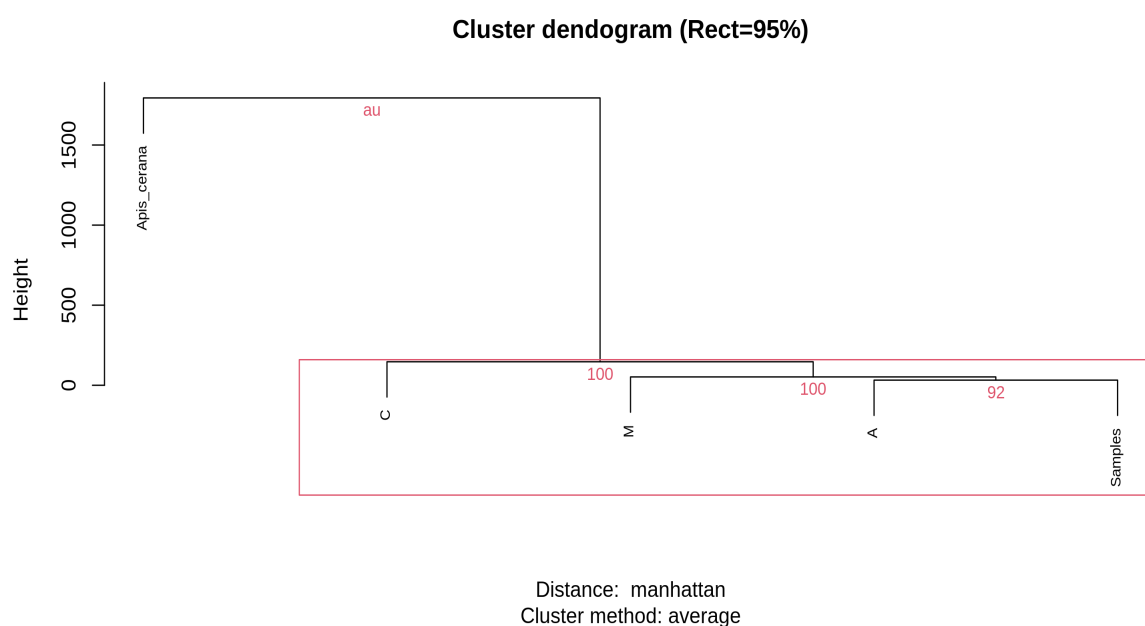


Figure 2. Cluster Dendrogram showing distance between sampled animals (“samples”) and with evolutionary branches A (African), M (Northern Europe)

and C (South-eastern European), accordingly with *Vg* sequences data, available on GenBank. *Apis cerana* is representing the external species from the same genus. Values on the dendrogram are AU p-values (Red), and clusterlabels (grey, bottom). AU $\geq 95\%$ are indicated by the red rectangles.



A total of 203 SNPs and 204 SNPs, were generated without missing-data, considering our approach per branch and country, respectively (see supplementary material: Table 3). We identified a pattern on the represented differences of the branches and countries. Showing that our samples are clustered with the evolutionary branch A (African) (Figure 2) and clustered with the bees sampled from Egypt and South Africa (Figure 1), which were the only African countries, as we would expect from an Africanized population. Both dendrograms showed clusters with AU $\geq 95\%$ and are considered to be strongly supported by data.

Little is known about the admixture rate in Brazil, to do so, it is necessary bearing in mind the elevate costs of carrying broad genetic and high-density

SNP surveys (as in Kadri et al., 2016 and Nelson et al. 2017). In Kadri et al. (2016) study (3,606,720 SNPs found in Africanized bees), using a regression model, the authors concluded that allele frequencies within Africanized bees were more correlated with A lineage allele frequencies (GML; $r=0.529$) in comparison with both M ($r=0.102$) and C ($r=-0.08$) lineage allele frequencies.

Chapman's et al. (2017) study, compared a 37 and 95 SNP panels for honeybee ancestry assignment that differentiate Africanized bees from commercial populations. The authors reported that the 37 SNP panel was significantly more cost-efficient (~60-70%) than the more extended panel.

Assessing *Vg* gene as a proxy towards a genetic passport for honeybees' subspecies origin, can contribute to unravel the admixture rate within the country. Also, in many countries, Africanized honeybees are not very appreciated due to its aggressive behavior and other considered undesired traits. If this gene and its previously identified SNPs could act as an indicator of subspecies clade, rapid and inexpensive surveys can be performed.

Another dimension is added by the deliberate replacement of subspecies in some regions by non-native bees with more desirable characters and a greater commercial interest (e.g., the replacement of *A. m. mellifera* in northern and central Europe by *A. m. carnica* or *A. m. ligustica*) (Bouga et al. 2011) or nearly introduction of European subspecies in Brazil to crossbreed and change some undesired traits (as aggressive behavior and tendency to abscond). The downside of these economically-driven processes is an increasing trend towards uniformity of honey bee populations across Brazil and/or Americas, and can lead to a loss of both genetic diversity and specific adaptations to local conditions (Meixner et al. 2010) as it happened before.

Thus, there is a widely recognized need to encourage national breeding efforts to preserve local adaptation and to maintain local strains in isolated conservation apiaries. To attain this goal, it is necessary to have a reference base to identify strains to be used for general assessments and breeding. To provide a stable baseline, it is important that this reference reflects the natural variation of honey bees, since beekeepers and breeders are known to manifest the tendency and desire to work with non-native stock.

References

- Alburaki, M., et al. (2011) Mitochondrial structure of eastern honeybee populations from Syria, Lebanon and Iraq. *Apidologie* 42 (5), 628–641.
- Amdam, G. V., et al. (2003) Disruption of vitellogenin gene function in adult honeybees by intra-abdominal injection of double-stranded RNA. *BMC Biotechnology*, 3 (1), 1-8.
- Bouga, M. et al. (2011) A review of methods for discrimination of honey bee populations as applied to European beekeeping. *Journal of Apicultural Research* 50 (1), 51-84.
- Chapman, N. C., et al. (2017) An abbreviated SNP panel for ancestry assignment of honeybees (*Apis mellifera*). *Apidologie*, 48 (6) 776-783.
- De la Rúa, P., et al. (2009) Biodiversity, conservation and current threats to European honeybees. *Apidologie* 40 (3), 263-284.
- Dogantzis, K. A., Zayed, A. (2019) Recent advances in population and quantitative genomics of honey bees. *Current Opinion in Insect Science* 31, 93-98.
- Ilyasov, R. A., et al. (2015) Nucleotide polymorphism of the gene *Vg* of honey bees. *Биомика* 7 (1), 54-61.

- Ilyasov, R. A., et al. (2020) A revision of subspecies structure of western honey bee *Apis mellifera*. Saudi Journal of Biological Sciences.
- Kadri, S. M., et al. (2016) A variant reference data set for the Africanized honeybee, *Apis mellifera*. Scientific data 3 (1), 1-6.
- Kent, C. F., et al. (2011) Adaptive evolution of a key gene affecting queen and worker traits in the honey bee, *Apis mellifera*. Molecular Ecology 20 (24), 5226-5235.
- Kerr, W. E. (1957) Introdução de abelhas africanas no Brasil. *Brasil Apicola* 3, 211–213.
- Kocher, S. D., Paxton, R. J. (2014) Comparative methods offer powerful insights into social evolution in bees. *Apidologie* 45 (3), 289-305.
- Meixner, M. D., et al. (2010) Conserving diversity and vitality for honey bee breeding. *Journal of Apicultural Research* 49 (1), 85-92.
- Meixner, M. D., et al. (2014) Honey bee genotypes and the environment. 183-187.
- Nelson, R. M., et al. (2017) Genomewide analysis of admixture and adaptation in the Africanized honeybee. *Molecular Ecology* 26 (14), 3603-3617.
- Purcell, S. et al (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum Genet.* 81(3), 559-575.
- Ruttner, F. (1988) *Biogeography and Taxonomy of Honeybees*. Springer Verlag. Berlin
- Sheppard, W. S., et al. (1997) *Apis mellifera ruttneri*, a new honey bee subspecies from Malta. *Apidologie*, 28 (5), 287-293.
- Sheppard, W. S., Meixner, M. D. (2003) *Apis mellifera pomonella*, a new honey bee subspecies from Central Asia. *Apidologie*, 34 (4), 367-375.

Whitfield, C. W., et al. (2006) Thrice out of Africa: ancient and recent expansions of the honey bee, *Apis mellifera*. *Science*, 314 (5799), 642-645.

Supplementary Material

Table 1. Information on the deposited sequences in GenBank used to generate the cluster dendrograms. Including the Species, Country, Branch, Accession ID and Total number of samples.

Species	Country	Branch	Accession List	Total
<i>Apis mellifera</i>	Egypt	-	JN557548.1; JN557547.1; JN557456.1; JN557455.1; JN557362.1; JN557361.1; JN557270.1; JN557269.1; JN557176.1; JN557175.1; JN557084.1; JN557083.1	12
	India	-	MH755913.1; MH755912.1; MH755878.1; MH755877.1; MH755843.1; MH755842.1; MH755808.1; MH755807.1; MH755773.1; MH755772.1; MH755738.1; MH755737.1	12
	Poland	-	JN557572.1; JN557571.1; JN557570.1; JN557569.1; JN557526.1; JN557525.1; JN557480.1; JN557479.1; JN557478.1; JN557477.1; JN557434.1; JN557433.1; JN557386.1; JN557385.1; JN557384.1; JN557383.1; JN557340.1; JN557339.1; JN557294.1; JN557293.1; JN557292.1; JN557291.1; JN557248.1; JN557247.1; JN557200.1; JN557199.1; JN557198.1; JN557197.1; JN557154.1; JN557153.1; JN557108.1; JN557107.1; JN557106.1; JN557105.1; JN557062.1; JN557061.1	36
	Russia	-	KU146557.1; KU146556.1; KU146555.1; KU146554.1; KU146553.1; KU146552.1; KU146551.1; KU146550.1; KU146549.1; KU146548.1; KU146547.1; KU146546.1; KU146545.1; KU146544.1; KU146543.1; KU146542.1; KU146541.1; KU146540.1; KU146539.1; KU146538.1; KJ645894.1; KJ645893.1; KJ645892.1; KJ645891.1; KJ645890.1; KJ645889.1; KJ645888.1; KJ645887.1; KJ645886.1; KJ645885.1; KJ645884.1; KJ645883.1; KJ572320.1; KJ572319.1; KJ572318.1; KJ572317.1; KJ572316.1; KJ572315.1; KJ572314.1; KJ572313.1; KJ572312.1; KJ572311.1; KJ572310.1; KJ572309.1; KJ572308.1; KJ572307.1; KJ572306.1; KJ572305.1; KJ572304.1; KJ572303.1; KJ572302.1; KJ572301.1; KJ572300.1; KJ572299.1; KJ572298.1; KJ572297.1; KJ572296.1; KJ572295.1; KJ572294.1; KJ572293.1; KJ572292.1; KJ572291.1; KJ572290.1; KJ572289.1; KJ572288.1; KJ572287.1; KJ572286.1; KJ572285.1; KJ532147.1; KJ532146.1; KJ532145.1; KJ532144.1; KJ532143.1; KJ532142.1; KJ532141.1; KJ532140.1; KJ532139.1; KJ532138.1; KJ532137.1; KJ532136.1; KJ532135.1; KJ532134.1; KJ532133.1; KJ532132.1; KJ532131.1; KJ532130.1; KJ532129.1; KJ532128.1; KJ532127.1; KJ532126.1; KJ532125.1; KJ532124.1	92
South Africa	A	JN557598.1; JN557597.1; JN557596.1; JN557595.1; JN557594.1; JN557593.1; JN557592.1; JN557591.1; JN557590.1; JN557589.1; JN557588.1; JN557587.1; JN557586.1; JN557585.1; JN557584.1; JN557583.1; JN557582.1; JN557581.1; JN557580.1; JN557579.1; JN557578.1; JN557577.1; JN557508.1; JN557507.1; JN557506.1; JN557505.1; JN557504.1; JN557503.1; JN557502.1; JN557501.1; JN557500.1; JN557499.1; JN557498.1; JN557497.1; JN557496.1; JN557495.1; JN557494.1; JN557493.1; JN557492.1; JN557491.1; JN557490.1; JN557489.1; JN557488.1; JN557487.1; JN557486.1; JN557485.1; JN557414.1; JN557413.1; JN557412.1; JN557411.1; JN557410.1; JN557409.1; JN557408.1; JN557407.1; JN557406.1; JN557405.1; JN557404.1; JN557403.1; JN557402.1; JN557401.1; JN557400.1; JN557399.1; JN557398.1; JN557397.1; JN557396.1; JN557395.1; JN557394.1; JN557393.1; JN557392.1; JN557391.1; JN557322.1; JN557321.1; JN557320.1; JN557319.1; JN557318.1; JN557317.1; JN557316.1; JN557315.1; JN557314.1; JN557313.1; JN557312.1; JN557311.1; JN557310.1; JN557309.1; JN557308.1; JN557307.1; JN557306.1; JN557305.1; JN557304.1; JN557303.1; JN557302.1; JN557301.1; JN557300.1; JN557299.1; JN557228.1; JN557227.1; JN557226.1; JN557225.1; JN557224.1; JN557223.1; JN557222.1; JN557221.1; JN557220.1; JN557219.1; JN557218.1; JN557217.1; JN557216.1; JN557215.1; JN557214.1; JN557213.1; JN557212.1; JN557211.1; JN557210.1; JN557209.1; JN557208.1; JN557207.1; JN557206.1; JN557205.1; JN557136.1; JN557135.1; JN557134.1; JN557133.1; JN557132.1; JN557131.1; JN557130.1; JN557129.1; JN557128.1; JN557127.1; JN557126.1; JN557125.1; JN557124.1; JN557123.1;	142	

		JN557122.1; JN557121.1; JN557120.1; JN557119.1; JN557118.1; JN557117.1; JN557116.1; JN557115.1; JN557114.1; JN557113.1	
Croatia	C	JN557524.1; JN557523.1; JN557522.1; JN557521.1; JN557520.1; JN557519.1; JN557518.1; JN557517.1; JN557516.1; JN557515.1; JN557432.1; JN557431.1; JN557430.1; JN557429.1; JN557428.1; JN557427.1; JN557426.1; JN557425.1; JN557424.1; JN557423.1; JN557338.1; JN557337.1; JN557336.1; JN557335.1; JN557334.1; JN557333.1; JN557332.1; JN557331.1; JN557330.1; JN557329.1; JN557246.1; JN557245.1; JN557244.1; JN557243.1; JN557242.1; JN557241.1; JN557240.1; JN557239.1; JN557238.1; JN557237.1; JN557152.1; JN557151.1; JN557150.1; JN557149.1; JN557148.1; JN557147.1; JN557146.1; JN557145.1; JN557144.1; JN557143.1; JN557060.1; JN557059.1; JN557058.1; JN557057.1; JN557056.1; JN557055.1; JN557054.1; JN557053.1; JN557052.1; JN557051.1	60
Egypt	C	JN557554.1; JN557553.1; JN557552.1; JN557551.1; JN557550.1; JN557549.1; JN557546.1; JN557545.1; JN557544.1; JN557543.1; JN557542.1; JN557541.1; JN557462.1; JN557461.1; JN557460.1; JN557459.1; JN557458.1; JN557457.1; JN557454.1; JN557453.1; JN557452.1; JN557451.1; JN557450.1; JN557449.1; JN557368.1; JN557367.1; JN557366.1; JN557365.1; JN557364.1; JN557363.1; JN557360.1; JN557359.1; JN557358.1; JN557357.1; JN557356.1; JN557355.1; JN557276.1; JN557275.1; JN557274.1; JN557273.1; JN557272.1; JN557271.1; JN557268.1; JN557267.1; JN557266.1; JN557265.1; JN557264.1; JN557263.1; JN557182.1; JN557181.1; JN557180.1; JN557179.1; JN557178.1; JN557177.1; JN557174.1; JN557173.1; JN557172.1; JN557171.1; JN557170.1; JN557169.1; JN557090.1; JN557089.1; JN557088.1; JN557087.1; JN557086.1; JN557085.1; JN557082.1; JN557081.1; JN557080.1; JN557079.1; JN557078.1; JN557077.1	72
Germany	C	JN557514.1; JN557513.1; JN557512.1; JN557509.1; JN557422.1; JN557421.1; JN557420.1; JN557415.1; JN557328.1; JN557327.1; JN557326.1; JN557325.1; JN557236.1; JN557235.1; JN557234.1; JN557229.1; JN557142.1; JN557141.1; JN557140.1; JN557137.1; JN557050.1; JN557049.1; JN557048.1; JN557041.1	24
Slovenia	C	JN557532.1; JN557531.1; JN557530.1; JN557529.1; JN557528.1; JN557527.1; JN557440.1; JN557439.1; JN557438.1; JN557437.1; JN557436.1; JN557435.1; JN557346.1; JN557345.1; JN557344.1; JN557343.1; JN557342.1; JN557341.1; JN557254.1; JN557253.1; JN557252.1; JN557251.1; JN557250.1; JN557249.1; JN557160.1; JN557159.1; JN557158.1; JN557157.1; JN557156.1; JN557155.1; JN557068.1; JN557067.1; JN557066.1; JN557065.1; JN557064.1; JN557063.1	36
Poland	M	JN557576.1; JN557575.1; JN557574.1; JN557573.1; JN557568.1; JN557567.1; JN557566.1; JN557565.1; JN557564.1; JN557563.1; JN557562.1; JN557561.1; JN557560.1; JN557559.1; JN557558.1; JN557557.1; JN557556.1; JN557555.1; JN557484.1; JN557483.1; JN557482.1; JN557481.1; JN557476.1; JN557475.1; JN557474.1; JN557473.1; JN557472.1; JN557471.1; JN557470.1; JN557469.1; JN557468.1; JN557467.1; JN557466.1; JN557465.1; JN557464.1; JN557463.1; JN557390.1; JN557389.1; JN557388.1; JN557387.1; JN557382.1; JN557381.1; JN557380.1; JN557379.1; JN557378.1; JN557377.1; JN557376.1; JN557375.1; JN557374.1; JN557373.1; JN557372.1; JN557371.1; JN557370.1; JN557369.1; JN557298.1; JN557297.1; JN557296.1; JN557295.1; JN557290.1; JN557289.1; JN557288.1; JN557287.1; JN557286.1; JN557285.1; JN557284.1; JN557283.1; JN557282.1; JN557281.1; JN557280.1; JN557279.1; JN557278.1; JN557277.1; JN557204.1; JN557203.1; JN557202.1; JN557201.1; JN557196.1; JN557195.1; JN557194.1; JN557193.1; JN557192.1; JN557191.1; JN557190.1; JN557189.1; JN557188.1; JN557187.1; JN557186.1; JN557185.1; JN557184.1; JN557183.1; JN557112.1; JN557111.1; JN557110.1; JN557109.1; JN557104.1; JN557103.1; JN557102.1; JN557101.1; JN557100.1; JN557099.1; JN557098.1; JN557097.1; JN557096.1; JN557095.1; JN557094.1; JN557093.1; JN557092.1; JN557091.1	108
Spain	M	JN557540.1; JN557539.1; JN557538.1; JN557537.1; JN557536.1; JN557535.1; JN557534.1; JN557533.1; JN557448.1; JN557447.1; JN557446.1; JN557445.1; JN557444.1; JN557443.1; JN557442.1; JN557441.1; JN557354.1; JN557353.1; JN557352.1; JN557351.1; JN557350.1; JN557349.1; JN557348.1; JN557347.1; JN557262.1; JN557261.1; JN557260.1; JN557259.1; JN557258.1; JN557257.1; JN557256.1; JN557255.1; JN557168.1; JN557167.1; JN557166.1; JN557165.1; JN557164.1; JN557163.1; JN557162.1; JN557161.1; JN557076.1; JN557075.1; JN557074.1; JN557073.1; JN557072.1; JN557071.1; JN557070.1; JN557069.1	48

MH755923.1; MH755922.1; MH755921.1; MH755920.1; MH755919.1; MH755918.1; MH755917.1; MH755916.1; MH755915.1; MH755914.1; MH755911.1; MH755910.1; MH755909.1; MH755908.1; MH755907.1; MH755906.1; MH755905.1; MH755904.1; MH755903.1; MH755902.1; MH755901.1; MH755900.1; MH755899.1; MH755898.1; MH755897.1; MH755896.1; MH755895.1; MH755894.1; MH755893.1; MH755892.1; MH755891.1; MH755890.1; MH755889.1; MH755888.1; MH755887.1; MH755886.1; MH755885.1; MH755884.1; MH755883.1; MH755882.1; MH755881.1; MH755880.1; MH755879.1; MH755876.1; MH755875.1; MH755874.1; MH755873.1; MH755872.1; MH755871.1; MH755870.1; MH755869.1; MH755868.1; MH755867.1; MH755866.1; MH755865.1; MH755864.1; MH755863.1; MH755862.1; MH755861.1; MH755860.1; MH755859.1; MH755858.1; MH755857.1; MH755856.1; MH755855.1; MH755854.1; MH755853.1; MH755852.1; MH755851.1; MH755850.1; MH755849.1; MH755848.1; MH755847.1; MH755846.1; MH755845.1; MH755844.1; MH755841.1; MH755840.1; MH755839.1; MH755838.1; MH755837.1; MH755836.1; MH755835.1; MH755834.1; MH755833.1; MH755832.1; MH755831.1; MH755830.1; MH755829.1; MH755828.1; MH755827.1; MH755826.1; MH755825.1; MH755824.1; MH755823.1; MH755822.1; MH755821.1; MH755820.1; MH755819.1; MH755818.1; MH755817.1; MH755816.1; MH755815.1; MH755814.1; MH755813.1; MH755812.1; MH755811.1; MH755810.1; MH755809.1; MH755806.1; MH755805.1; MH755804.1; MH755803.1; MH755802.1; MH755801.1; MH755800.1; MH755799.1; MH755798.1; MH755797.1; MH755796.1; MH755795.1; MH755794.1; MH755793.1; MH755792.1; MH755791.1; MH755790.1; MH755789.1; MH755788.1; MH755787.1; MH755786.1; MH755785.1; MH755784.1; MH755783.1; MH755782.1; MH755781.1; MH755780.1; MH755779.1; MH755778.1; MH755777.1; MH755776.1; MH755775.1; MH755774.1; MH755771.1; MH755770.1; MH755769.1; MH755768.1; MH755767.1; MH755766.1; MH755765.1; MH755764.1; MH755763.1; MH755762.1; MH755761.1; MH755760.1; MH755759.1; MH755758.1; MH755757.1; MH755756.1; MH755755.1; MH755754.1; MH755753.1; MH755752.1; MH755751.1; MH755750.1; MH755749.1; MH755748.1; MH755747.1; MH755746.1; MH755745.1; MH755744.1; MH755743.1; MH755742.1; MH755741.1; MH755740.1; MH755739.1; MH755736.1; MH755735.1; MH755734.1; MH755733.1; MH755732.1; MH755731.1; MH755730.1; MH755729.1; MH755728.1; MH755727.1; MH755726.1; MH755725.1; MH755724.1; MH755723.1; MH755722.1; MH755721.1; MH755720.1; MH755719.1; MH755718.1; MH755717.1; MH755716.1; MH755715.1; MH755714.1; JN557511.1; JN557510.1; JN557417.1; JN557416.1; JN557324.1; JN557323.1; JN557231.1; JN557230.1; JN557139.1; JN557138.1; JN557043.1; JN557042.1;

Table 2. Number of sequences accessed per country and exons (2-7) of the gene Vg, available on GenBank database.

Country	Branch	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Total
Croatia	C	10	10	10	10	10	10	60
Egypt	-	2	2	2	2	2	2	12
Egypt	C	12	12	12	12	12	12	72
Germany	C	4	4	4	4	4	4	24
India	-	2	2	2	2	2	2	12
Poland	-	6	6	6	6	6	6	36
Poland	M	18	18	18	18	18	18	108
Russia	-	12	12	22	22	12	12	92
Slovenia	C	6	6	6	6	6	6	36
South_Africa	A	24	24	24	22	24	24	142
Spain	M	8	8	8	8	8	8	48
Total		104	104	114	112	104	104	642

Table 3. List of variants and correspondent alleles. GenBank ID accordingly with the genome reference sequence (CHROM), respective position (POS), per Branch (A, C, M); Country (Croatia, Egypt, Germany, India, Poland, Russia, Slovenia, South Africa and Spain); External species (*Apis cerana*) and Samples (Africanized honeybees sampled in our study).

CHROM	POS	Branch					Country										
		A	<i>Apis cerana</i>	C	M	Samples	<i>Apis cerana</i>	Croatia	Egypt	Germany	India	Poland	Russia	Samples	Slovenia	South Africa	Spain
NC_037641.1	5029670	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5029685	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5029689	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5029703	G	G	G	A	G	G	G	G	G	G	A	A	G	G	G	A
NC_037641.1	5029706	G	C	G	G	G	C	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5029730	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5029760	G	G	G	A	G	G	G	A	G	G	A	A	G	G	G	A
NC_037641.1	5029781	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5029787	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5029808	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5029811	A	T	A	A	A	T	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5029845	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5029877	T	G	T	T	T	G	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5029884	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5029890	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5029955	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5029962	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5029984	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5029994	G	T	G	G	G	T	G	G	G	G	G	G	G	G	G	G

NC_037641.1	5030006	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030026	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030030	A	G	A	A	A	G	A	A	A	G	G	A	A	A	A	A
NC_037641.1	5030277	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030278	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030302	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030304	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030310	T	T	C	T	T	T	C	T	C	C	T	T	T	C	T	T
NC_037641.1	5030314	C	G	C	C	C	G	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030317	A	T	A	A	A	T	T	A	A	T	A	A	A	A	A	A
NC_037641.1	5030320	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5030332	C	T	T	C	C	T	T	C	T	T	C	C	C	T	C	C
NC_037641.1	5030336	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5030343	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5030351	C	G	C	C	C	G	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030356	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030359	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5030375	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030380	T	A	T	T	T	A	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5030385	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5030388	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030404	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030409	C	C	T	T	C	C	C	T	T	C	T	T	C	T	C	T
NC_037641.1	5030430	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030441	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5030489	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030491	A	C	A	A	A	C	A	A	A	A	A	A	A	A	A	A

NC_037641.1	5030500	G	T	G	G	G	T	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5030511	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030523	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5030542	G	C	G	G	G	C	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5030545	T	C	C	T	C	C	C	T	C	C	T	T	C	C	T	T
NC_037641.1	5030564	A	G	G	A	A	G	G	A	G	G	A	A	A	G	A	A
NC_037641.1	5030820	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030831	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030853	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5030870	C	G	C	C	C	G	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030876	A	C	A	A	A	C	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5030880	C	A	C	C	C	A	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030897	T	A	T	T	T	A	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5030910	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5030912	A	T	A	A	A	T	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5030916	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5030942	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030972	C	G	C	C	C	G	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030973	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030996	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5030999	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031006	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031042	G	C	G	G	G	C	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5031077	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031079	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5031080	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031083	A	A	G	A	A	A	G	A	G	G	A	A	A	G	A	A

NC_037641.1	5031084	T	C	C	T	T	C	C	T	C	C	T	T	T	C	T	T
NC_037641.1	5031096	A	A	T	A	A	T	T	A	T	T	A	A	A	T	A	A
NC_037641.1	5031097	G	T	G	G	G	T	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5031105	C	G	C	C	C	G	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031110	G	C	G	G	G											
NC_037641.1	5031122	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5031129	C	C	T	C	C	C	C	C	T	C	C	C	C	T	C	C
NC_037641.1	5031130	C	C	T	C	C	C	C	C	T	T	C	C	C	T	C	C
NC_037641.1	5031131	T	G	T	T	T	G	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5031138	C	C	T	C	C	C	C	T	T	T	C	C	C	T	C	C
NC_037641.1	5031170	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031180	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031194	G	C	G	G	G	C	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5031236	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031241	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5031252	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5031269	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031270	G	C	G	G	G	C	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5031271	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5031293	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031322	C	C	C	T	T	C	C	C	C	C	T	T	T	C	C	T
NC_037641.1	5031336	C	G	C	C	C	G	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031348	C	G	C	C	C	G	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031350	G	A	A	G	G	A	G	G	A	A	G	G	G	A	G	G
NC_037641.1	5031360	A	T	A	A	A	T	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5031386	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031392	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C

NC_037641.1	5031396	A	A	T	A	T	T	T	T	T	T	A	A	T	T	A	A
NC_037641.1	5031420	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031425	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031445	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031468	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031505	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5031526	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5031561	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5031565	A	T	A	A	A	T	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5031567	C	A	C	C	C	A	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031571	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5031581	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5031601	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031624	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5031626	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031630	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031632	C	A	C	C	C	A	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031639	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031642	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5031652	G	G	C	C	G	G	G	G	C	G	C	C	G	C	G	C
NC_037641.1	5031657	A	G	A	A	A	G	G	A	A	G	A	A	A	A	A	A
NC_037641.1	5031660	G	T	G	G	G	T	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5031661	T	A	T	T	T	A	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5031664	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5031665	G	C	G	G	G	C	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5031670	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031676	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G

NC_037641.1	5031695	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5031707	T	A	T	T	T	A	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5031708	T	A	T	T	T	A	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5031722	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5031724	G	C	G	G	G	C	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5031738	T	G	T	T	T	G	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5031740	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5031753	C	A	C	C	C	A	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031795	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031822	C	A	C	C	C	A	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5031825	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5031859	A	T	A	A	A	T	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5031900	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5031917	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5032790	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5032799	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5032850	A	A	A	A	G	A	G	A	G	G	A	A	G	G	A	A
NC_037641.1	5032858	T	G	T	T	T	G	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5032940	T	A	T	T	T	A	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5033021	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5033045	A	C	A	A	A	C	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5033068	A	C	A	A	A	C	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5033071	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5033132	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5033161	T	A	T	T	T	A	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5033180	A	G	A	A	G	G	G	A	G	G	A	A	G	G	A	A
NC_037641.1	5033195	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A

NC_037641.1	5033200	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5033204	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5033252	G	C	G	G	G	C	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5033281	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5033282	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5033309	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5033321	G	C	G	G	G											
NC_037641.1	5033322	C	T	C	C	C											
NC_037641.1	5033324	C	T	C	C	C											
NC_037641.1	5033332	T	C	T	T	T											
NC_037641.1	5033662	G	G	A	G	G	G	A	G	A	A	G	G	G	A	G	G
NC_037641.1	5033668	C	C	T	C	C	C	T	C	T	T	C	C	C	T	C	C
NC_037641.1	5033710	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5033725	A	C	A	A	A	C	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5033737	G	G	A	G	G	G	A	A	A	A	G	G	G	A	G	G
NC_037641.1	5033741	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5033743	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5033753	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5033755	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5033758	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5033781	T	A	T	T	T	A	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5033798	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5033842	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5033864	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5033881	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5033896	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5033937	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C

NC_037641.1	5033955	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5033990	T	A	T	T	T	A	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5033995	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5034044	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5034056	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5034067	T	C	T	T	T	C	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5034076	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5034103	G	C	G	G	G	C	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5034110	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5034133	T	A	T	T	T	A	T	T	T	T	T	T	T	T	T	T
NC_037641.1	5034178	A	G	A	G	G	G	A	A	A	A	A	G	G	A	A	G
NC_037641.1	5034187	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5034202	G	T	G	G	G	T	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5034228	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5034235	C	G	C	C	C	G	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5034239	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5034599	G	G	A	A	G											
NC_037641.1	5034641	G	G	A	G	G											
NC_037641.1	5034847	C	T	C	C	C	T	C	C	C	C	C	C	C	C	C	C
NC_037641.1	5034883	G	A	G	G	G	A	G	G	G	G	G	G	G	G	G	G
NC_037641.1	5034959	A	G	A	A	A	G	A	A	A	A	A	A	A	A	A	A
NC_037641.1	5035112	C	T	T	T	C	T	T	T	T	T	T	T	C	T	C	C
NC_037641.1	5030413						G	A	G	G	A	G	G	G	G	G	G
NC_037641.1	5030428						G	A	G	G	A	G	G	G	G	G	G
NC_037641.1	5030524						G	A	G	G	A	G	G	G	G	G	G
NC_037641.1	5033162						G	G	G	T	T	G	G	G	T	G	G
NC_037641.1	5034220						C	C	C	C	C	C	C	C	C	C	T

NC_037641.1	5034223	G	G	G	G	G	G	A	G	G	G	A
NC_037641.1	5034674	G	G	G	G	G	G	A	G	G	G	A
NC_037641.1	5035064	T	T	T	T	T	T	T	T	T	T	C

CAPÍTULO VI

Considerações finais

CAPÍTULO VI - Considerações Finais

Em síntese geral da tese, pode-se concluir a extrema relevância do gene da vitelogenina quando considerado nos processos e performance reprodutiva de abelhas. O gene em causa foi amplamente estudado em outras espécies de insetos mas continua a ter uma componente enigmática nas abelhas. Especialmente a nível estrutural.

Características correlacionadas como o peso à emergência, o peso dos ovários e o número de ovários apresentam um efeito relevante no desempenho reprodutivo das rainhas. Assim, informações sobre aspetos genéticos de características morfométricas de rainhas, que sejam relacionadas com o aumento da produtividade da colônia são de extrema importância para auxiliar no desenvolvimento de programas de seleção. Nesse contexto, este trabalho vem corroborar o efeito do gene *Vg* no carácter morfométrico (peso à emergência), o que permitirá conduzir futuras abordagens focadas em características alvo que sejam mais específicas em termos de performance reprodutiva, como o peso dos ovários e número de ovariolos. A utilização de características morfométricas como critérios de seleção devem continuar a ser alvo de estudo e desenvolvimento, pois até à data, pouco se sabe.

É importante realçar a necessidade do encorajamento por parte dos esforços nacionais, para que seja estabelecido um programa de melhoramento visando a preservação da espécie em território nacional. É necessário que se continue a desvendar a origem das abelhas africanizadas nas várias regiões do Brasil, uma vez que ainda permanece enigmática em muitas regiões. Para atingir esse objetivo, é necessário ter uma base de referência para a identificação dos *branches* evolutivos, especialmente se se visar o melhoramento genético da espécie. Para fornecer uma linha de base estável, é importante acessar a diversidade de ecótipos e sua distribuição nas diferentes regiões do país. Conhecer a distribuição e linhagens, contribuirá para inferir sobre a história da africanização no Brasil.

Na perspectiva de programas de melhoramento focados na apicultura, sabe-se que a avaliação genética tem base fundamentada em modelos

estatísticos que são os pilares dessa ciência. Contudo, as informações fisiológicas combinadas com dados moleculares, permitem um melhor entendimento das características em foque e conseqüentemente maior possibilidade de avaliação genética mais acurada. Então, este trabalho prova-nos que ambas as ciências devem andar de mãos dadas para ampliar o espectro de discussão e raciocínio no que toca à criação de programas de melhoramento que visam impulsionar a pesquisa e apicultura.