Short Note [Nota corta]

CASTE DEVELOPMENT IN HYMENOPTERA, A MOLECULAR VIEW FOR FARMING IMPROVEMENT OF STINGLESS BEES †

[DESARROLLO DE CASTAS EN HIMENÓPTERA, UNA VISIÓN MOLECULAR DEL CULTIVO DE ABEJAS SIN AGUIJÓN]

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SUMMARY

Background: The evolution and genetic elements for the development of the caste is an enigma in Hymenoptera, as well as the total impact that the genetic elements given in the food of the larvae have on the development of bees. During the last decades, micro RNAs (miRNAs) from nurse bee secretions in larval food have been found to constitute an additional element in the regulatory control of caste determination. Furthermore, social differentiation is a complex developmental process influenced by genetic, epigenetic, and environmental factors. **Objective:** To examine and summarize miRNAs as a regulatory component of larval food with an effect on caste development in eusocial hymenoptera. **Methodology.** The sequences of miRNAs expressed in eusocial bees were obtained from miRBase.org. MUSCLE V.3 enabled alignment and correction was carried out using Fast.Dist. The TreeDyn software was used to obtain the phylogenetic trees. **Results:** The role of diet and the relationship with microRNAs in caste differentiation and regulation may be part of a general mechanism that involves selective plant genetic information necessary for insect development. **Implications:** The in vitro cultivation of stingless bees is a practice that is already carried out in some species of commercial importance; However, studies that demonstrate how development, differentiation and regulation of gene expression would be shaping the differentiation of different castes are still gaps that must begin to be addressed. In vitro breeding would require the study of environmental and molecular variables that would modulate the expression of the castes. **Conclusion**: The biogenesis, regulation and functions of microRNA in Eukarya are still obscure, however, some light has been shed on the molecular basis of caste differentiation in eusocial bees modulated by 2 miRN transfer pathways; endogenous and exogenous.

Key words: eusociality; caste determination; microRNA; epigenetic regulation.

RESUMEN

Antecedentes: La evolución y los elementos genéticos para el desarrollo de la casta es un enigma en Himenópteros, así como el impacto total que tienen los elementos genéticos dados en el alimento de las larvas en el desarrollo de las abejas. Durante las últimas décadas, los micro ARN (miARN) de las secreciones de las abejas nodrizas en los alimentos para las larvas constituyen un elemento adicional en el control regulatorio de la determinación de castas. Además, esta diferenciación social es un proceso de desarrollo complejo influenciado por factores genéticos, epigenéticos y ambientales. **Objetivo:** Examinar y resumir los miARN como un

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componente regulador del alimento de las larvas con efecto sobre el desarrollo de castas en himenópteros eusociales. **Metodología**. Las secuencias de miRNAs expresados en abejas eusociales fueron obtenidos a partir de miRBase.org. MUSCLE V.3 permitió el alineamiento y corrección mediante Fast.Dist. El software TreeDyn fue utilizado para obtener los arboles filogenéticos. **Resultados:** El papel de la alimentación y la relación con los microARN en la diferenciación y regulación de castas puede ser parte de un mecanismo general que involucra información genética vegetal selectiva necesaria para el desarrollo de los insectos. **Implicaciones:** El cultivo *in vitro* de abejas sin aguijón es una práctica que se realiza en algunas especies de importancia comercial; Sin embargo, los estudios que demuestren cómo el desarrollo, la diferenciación y la regulación de la expresión génica estarían modelando la diferenciación de las distintas castas son todavía lagunas que deben empezar a abordarse. La cría *in vitro* requerirá el estudio de variables ambientales y moleculares que modularían la expresión de las castas. **Conclusión:** La biogénesis, la regulación y las funciones del microARN en Eukarya aún son oscuras, sin embargo, se ha arrojado algo de luz sobre las bases moleculares de la diferenciación de castas en las abejas eusociales moduladas por dos vías de transferencia de miRN; endógena y exógena.

Palabras clave: eusocialidad; determinación de casta; microARN; regulación epigenética.

INTRODUCTION

Organized insect societies show a pattern of hierarchies and orderly distribution of work. Therefore, success is reflected in their communities where sociability is key to improving the inclusive attitude of an individual. In colonies of social insects, the organization in the distribution of work is clear, as well as characteristic and distinctive phenotypes within the same sex according to the role played. Highly eusocial (actual society) occurs and has evolved several times in Hymenoptera insects. Genetic analyses provide a new empirical baseline for understanding this evolution and the definition of castes in a more complex way than is handled today by referring to the caste of social insects merely as "the reproductive division of labor" and as "the division of labor among females based on reproductive specialization" (Michener, 1974; Sumner *et al.*, 2018; Hughes *et al.*, 2003).

Epigenetic mechanisms that affect gene expression and biological function have already been documented in eusocial bees, specifically through gene silencing mediated by short-chain non-coding RNA (Yan *et al.*, 2014; Søvik *et al.*, 2015; Ashby *et al.*, 2016; Villagra and Frías-Lasserre, 2020). MicroRNAs (miRNAs) are ncRNAs whose documented function is to regulate gene expression (Djuranovic *et al.*, 2011). miRNAs of 19-24 nucleotides in length, restricting protein production by degrading mRNA and inhibiting translation (Huntzinger and Izaurralde, 2011) and affecting multiple developmental and phenotype characteristics (Selbach *et al.*, 2008). The potential role of miRNAs in caste development has been studied and shown that miRNAs are expressed differentially during development. The meliponini tribe does not record to date any such study. The morphological differentiation of castes is a different process influenced by the food provided under a highly organized process of sourcing food in brood cells, resulting in different morphological, physiological, and behavioral phenotypes in the castes.

The active components that determine the fate of bee development remain elusive and even controversial (Buttstedt *et al.*, 2016). According to Sumner *et al.* (2006) and Solenn *et al.* (2015), the ability of a genotype to alter its ontogeny in response to an environmental change is due to its phenotypic plasticity during development. The roles of miRNAs in morphological and phenotypic plasticity are now a focus of study. However, there needs to be more information on how this influences behavioral phenotypic plasticity. The phenotypic plasticity of natural behavior due to the division of labor (DOL) characterized in *Apis mellifera* has been the model for understanding the genetic mechanisms that affect different behavioral phenotypes. Caste determination in most bees is caused by different stimuli in the quantity and quality of larval food (royal jelly) (*Apis mellifera*, Apini, Apidae).

In contrast, in (Meliponini, Apidae, except in the genus *Melipona*), the caste is determined by the amount of larval food (Nogueira-Neto, 1997). For this reason, it is important to show the regulatory mechanisms of environmentally mediated gene expression in the development of the caste in stingless bees, which is mainly attributed to the amount of food and not the components. Here we reviewed the literature comprising the role of microRNAs on caste differentiation and regulation of development in eusocial bees. The production of honey from stingless bees has been carried out since ancient times. Maya used *Melipona beecheii* to obtain several products that have gained acceptance in current times, therefore the production of queens to increase productions would welcome more information for this process.

METHODOLOGY

A literature analysis was performed on reports of Endogenous and Exogenous miRNAs expressed in eusocial bees. Those with influence on the determination of caste and consequently on fertility were selected. The sequences of the selected miRNAs were obtained using the miRBase.org database, this is a searchable database of published miRNA sequences and annotations. The structure of miRNAs was visualized and documented using the RNA structure tool; it is a complete package for the prediction and analysis of RNA secondary structures. The functions of each one were analyzed according to records published in the literature, specifically those model organisms of Exogenous (*Arabidopsis thaliana*) and Endogenous (*Drosophila melanogaster and A. mellifera*) miRNAs.

To search for sequence homologies, the BLAST of the miRBase platform was used, the alignments were carried out using the MUSCLE V.3 program and the alignment corrections were made using Fast.Dist. The graphical representation and editing of the phylogenetic trees was carried out with the TreeDyn tool. The programs used were free access, the selection of these is because they allowed better visualization of the similarities in the sequences and to investigate the functions. Drawings were carried out in Microsoft PowerPoint 2010 and Adobe Photoshop Elements 9.

RESULTS AND DISCUSSION

Biogenesis of microRNA

miRNAs are small molecules of non-coding RNA (ncRNA), consisting of around 20-24 nucleotides and with a characteristic hairpin-shaped structure (Lee *et al.*, 2004; Mohr and Mott, 2015; O'Brien *et al.*, 2018). With a phosphate group at the 5' end and a hydroxyl group at the 3' end, these are derived from a process that arises in the nucleus and ends in the cytoplasm. Then ncRNA carries out their function (Mohr and Mott, 2015); either in the development, apoptosis, cell differentiation, reproduction, behavior, and physiology in eukaryotes, including plants and animals (Ledda *et al.*, 2020). They play a significant regulatory role in animals and plants by binding to messenger RNAs (mRNA) and inhibiting their translation into protein.

MicroRNA gene transcription

The initial step in microRNA biogenesis is the transcription of miRNA genes. For example, in Drosophila melanogaster, these are transcribed in the nucleus by RNA polymerase II, thus facilitating the transcription of miRNA loci in these related species. RNA polymerase II produces a long chain of RNA due to the transcript known as primary microRNA (primiRNA), which is several kb long (Schanen and Li 2011; Lucas and Raikhel, 2013; Ylla *et al.*, 2016). MicroRNA loci organization can be a single transcript unit containing a single miRNA gene or, in some cases, organized as a polycistronic transcript unit containing more than one miRNA. Though originally as RNA Pol III transcripts, miRNAs are loaded with the canonical 5' 7-methyl guanosine caps and 3' polyadenylation, showing clear evidence of RNA Pol II-mediated transcription (Lee *et al.*, 2002; Rodriguez *et al.*, 2004; Lucas and Raikhel, 2013b;).

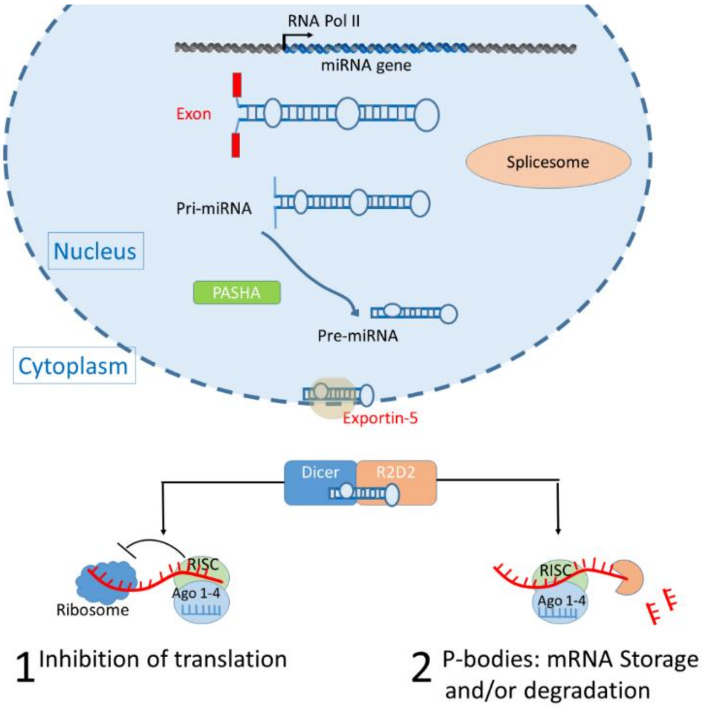

Figure 1. Biogenesis of miRNAs in *D. melanogaster*. microRNAs are transcribed by RNA Pol II at the first place and then a serial step from nucleolus to cytoplasm confers a mature set of microRNAs.

Processing of pri-miRNAs

The primiRNAs form hairpin-shaped structures with a double-stranded stem and a terminal loop that must subsequently be processed to obtain the mature miRNA molecule. In *D. melanogaster*, primiRNAs undergo site-specific processing mediated by the Microprocessor complex, a multi-protein complex composed of an RNase III family and a double-strand RNA binding protein, Drosha, and Pasha, respectively (Denli *et al.*, 2004; Gregory *et al.*, 2004). The miRNA, after transcription, is end methylated before being exported from the nucleus to the cytoplasm. This first step yields a 70 nt long microRNA. After their export to the cytoplasm, the second processing step is mediated by another protein complex related to an RNase III, Dicer. Dicer requires another RNA binding protein in order to mediate the final processing step yielding a mature 22 nt long microRNA (György *et al.*, 2001; Ketting *et al.*, 2001; W and L., 2001) (Figure 1).

microRISC Components

In order to be fully functional, mature microRNAs form part of the RNA-induced silencing complex (RISC). In the cytoplasm, the miRNA-RISC (miRISC) protein Argonaute (AGO) selects one of the miRNA strands to complete the gene silencing and degradation process. miRISC is a well-conserved complex ranging between 200 and 400 kDa depending on the organism (Lucas and Raikhel, 2013b; Santhekadur and Kumar, 2019). Argonaute proteins are a well-documented family of RNA-binding proteins mediating the RNA silencing process, widely disseminated to almost all organisms (Hutvagner and Simard, 2008). The miRISC acts recognizing and by a complementary binding with the microRNA, an mRNA is degraded, or their protein translation is limited by this process, (Tomoyasu *et al.*, 2008; Gu and Knipple, 2013; Santhekadur and Kumar 2019). These processes in which all these factors are affected by miRNA can be seen in figure 2, these factors affect in general in all multicellular organisms.

Regulation of functions by microRNA in Hymenoptera

miRNAs function as developmental switches (Stefani and Slack, 2008), that regulate gene expression in the nervous system and give rise to behavioral characteristic and phenotypes (Perkins *et al.*, 2007; Greenberg *et al.*, 2012). One of the most exciting aspects of miRNA affecting phenotypes are plant miRNAs. There are reports of plant miRNAs ingested from plant food sources that can pass through the gastrointestinal tract, enter the blood, accumulate systemically, and regulate gene expression in animals. The data shows gene regulation can be crossed between kingdoms and mediated by exogenous miRNAs. Once a new microRNA integrates into an animal's genetic regulatory network, it is usually retained and becomes challenging to lose during evolution (Heimberg *et al.*, 2008).

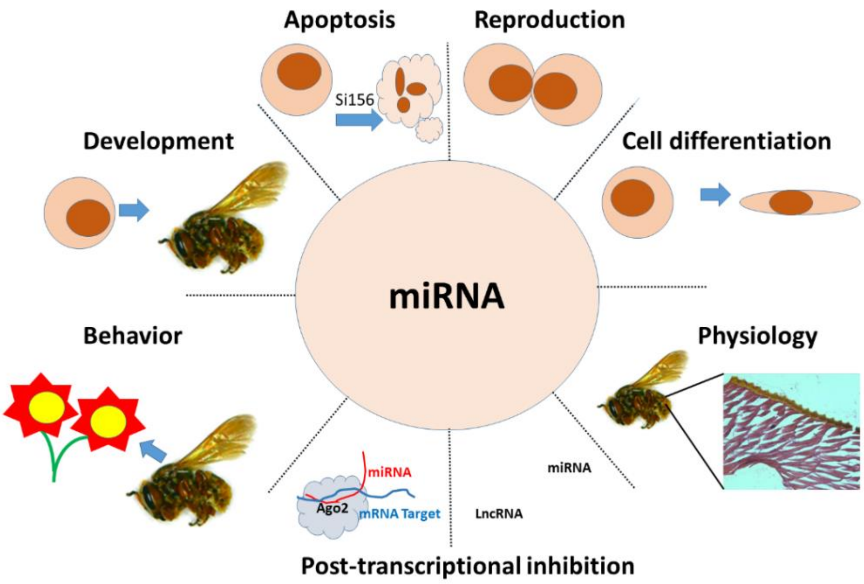

Figure 2. Functions of miRNAs in eukaryotes**.** The general aspects of microRNA regulation and their implications on different developmental processes.

As reported by Collins *et al.* (2017) miRNAs are associated with caste determination and differentiation in a relatively early process in eusocial evolution, and the role of individual miRNAs is generally not preserved as eusocial evolution progresses. They validated two miRNAs (Bte-miR-6001-5p and Bte-miR-6001-3p) expressed more in late-stage larvae intended for queens than in workers. In addition, the role in caste determination for miR-6001-5p and miR-6001-3p plays a regulatory role of Bte-miR-6001-5p as it includes the ecdysone-induced protein and ferredoxin. These proteins are associated with ecdysone responses in *Drosophila* (Palandri *et al.*, 2015). In *A. mellifera* the ecdysone is an ecdysteroid, that mediates several processes, including caste determination in eusocial bees (Rachinsky *et al.*, 1990) and primitively eusocial *Bombus terrestris* (Hartfelder *et al.*, 2000). Therefore, it is necessary to do additional studies. To show how endogenous miRNAs affect caste, it is also necessary to consider what mechanisms are conserved that explain eusociality in some Hymenoptera.

Greenberg and collaborators found that certain miRNA loci are specific to Hymenoptera, but are present only in eusocial taxa, or at least absent in non-eusocial wasps *Niphona longicornis* and *Nasonia vitripenni* (Greenberg *et al.*, 2012*)*. Therefore, it raises the possibility that some of these miRNAs are involved in the eusociality in Hymenoptera, as raised by Bonasio *et al.* (2010), where they show differences in the expression of miRNA between different species of eusocial ants, miRNA sequence data have advantages for estimating phylogenetic relationships.

In the Insecta class and the Order Hymenoptera specifically, it is evident that miRNAs miR-14-3P, miR-6001-5p and miR-6001-3p are conserved.

The genome sequence of the wasp *N. vitripennis* (Kent *et al.*, 2015), shows that during the evolution of sociality, the genes that were subject to selection in solitary ancestors co-opted to its use in caste-specific functions Figure 3. In the case of the ant *Dinoponera quadriceps*, it presents a simple society where individuals retain the ability to change phenotypes; however, as reported by Patalano *et al.* (2015) evidenced sequence homology in reproductive and worker ants.

In the case of miR-6001-5p and miR-6001-3p, both share the same functions in *A. mellifera,* regulating reproductive differentiation processes in queen bees; however, in *B. terrestris*, miR-6001-3p differs by its expression only in the cuticle of larvae destined for queens (Collins *et al.*, 2017).

According to the phylogeny evidenced from miR-6001-5p, the sequences are conserved in four species of Hymenoptera and one species of Diptera. It shows a more significant similarity between the sequences of *A. mellifera* with *D. quadriceps*. This similarity of sequence and common points in evolution may share their miRNAs' function Figure 4 and 5.

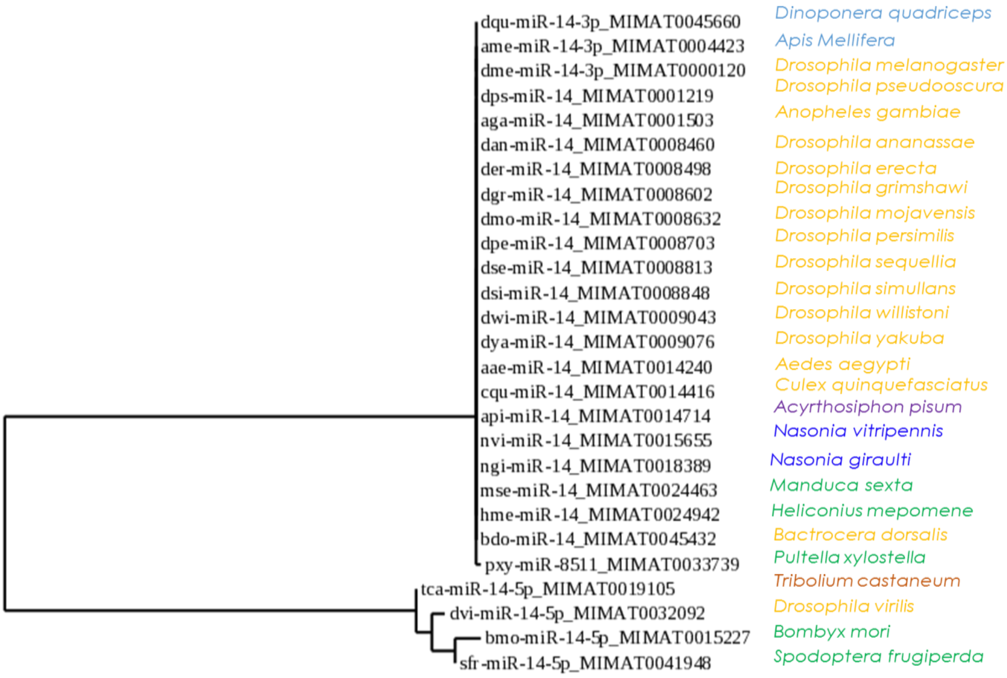

Figure 3. Phylogeny. Showing the homology of miR-14-13p in species of the Insecta class and represented in blue of the specific Order Hymenoptera. Created phylogeny program CrustalW2 (Madeira *et al.,* 2022). https://www.ebi.ac.uk/jdispatcher/

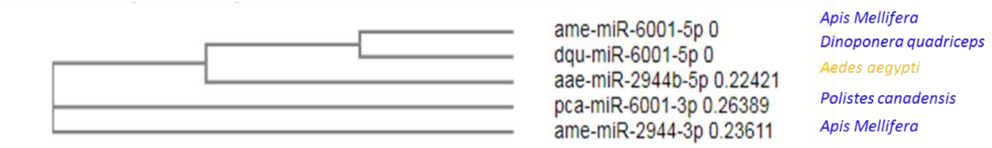

Figure 4. Phylogeny**.** Showing the homology of miR-6001-5p with other insects and the vast majority of the Order Hymenoptera. Created phylogeny program CrustalW2 (Madeira *et al.,* 2022). https://www.ebi.ac.uk/jdispatcher/

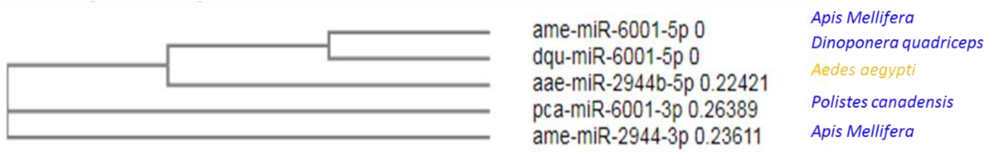

Figure 5. Phylogeny**.** Showing the homology of miR-6001-3P with other species of the order Hymenoptera. Created phylogeny program CrustalW2 (Madeira *et al.,* 2022). https://www.ebi.ac.uk/jdispatcher/

Regarding the homology of miR-6001-3p with other organisms of the Insecta class, it does not show any homology. However, it does show homology with the primitively eusocial wasp Polistes Canadensis, possibly sharing functions in caste differentiation (Figure 5).

Differentiation of castes in stingless bees: microRNAs and eusocial evolution

Insects provide an ideal model system to study the role of epigenetics in environmentally induced phenotypic evolution, as they have conserved chromatin, a human-like modification system, and most respond to environmental cues. The environment regulates epigenetic mechanisms and affects gene expression, acting as a mediator of changes in the phenotype induced by diet, which in larval stages changes gene expression, which in turn influences castes (Cridge *et al.*, 2015). According to Hartfelder *et al.* (2000), Sumner *et al.* (2006), Barchuk *et al.* (2007) and Collins *et al.* (2017), in a colony of *A. mellifera* caste determination is a decisive factor in the regulation of social behavior, it was closely related to the quality of honey. The food source thus affects gene expression together with epigenetic factors (Figure 2).

Nectar and pollen phytochemicals on honeybees may be widespread among social Hymenoptera or may be unique to the highly eusocial honeybee (Mao *et al.*, 2015). Further increasing the controversy over the impact of diet on the evolution of eusociality. Caste development in social insects represents the most important evolutionary transition from one level of organization to another and is believed to be critical to the ecological success of social insects (Zhu *et al.*, 2017).

In highly eusocial bee colonies, there are two sexes (males and females) and two castes (workers and queens). In social insects, the term caste is used more frequently to refer to the division of labor between females, based on reproductive specialization (Michener, 1974, 2007). Reproductive specialization is reflected in workers and queens being morphologically and physiologically different. The queens are dedicated to reproduction, so they have lost all the structures for food gathering and nest building. The workers dedicate themselves to collecting food and different activities within the nest. In some species, the workers are capable of reproduction, but since they are unable to mate, they are only capable of producing unfertilized (male) eggs.

Stingless bees present different mechanisms for caste determination; one of these is trophic determination, which can be found in almost all genera except Melipona (Sakagami, 1982). In this type of determination, the fate of the larva depends on nutritional differentiation; the larva that becomes the queen is placed in cells with more food than the workers. There are also two other variants of the caste-determining trophic system. The first consists of the construction of queen cells in advance. That is, the constructed cells are made exclusively to raise a queen. In these species, the queen cells are normally located on the periphery (Smith *et al.*, 2008; Smith and Suarez, 2010). In the second variant of trophic determination, queens are created in worker cells through emergency queen rearing; in this variant, enlarged queen cells are produced by the fusion of two adjoining worker cells (Wei *et al.*, 2019). While in the *Melipona* genus, caste determination depends on the individual's genotype, the effect of food is essential for its expression, defined as trophogenetics (Lisboa *et al.*, 2005; Lu *et al.*, 2021) depicted in Figure 6.

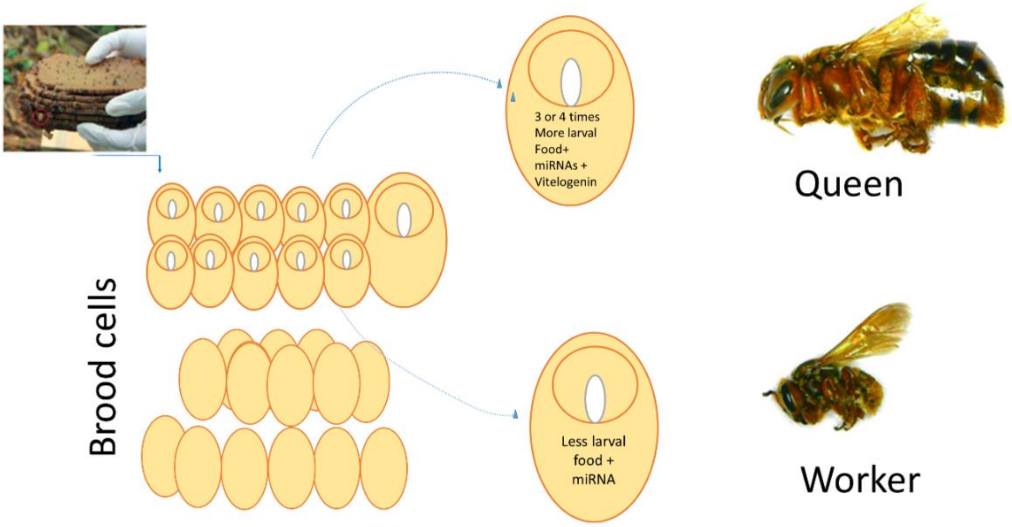

Figure 6. Determination of trophic caste is present in the Meliponini tribe, except Melipona. In this type of differentiation, the fate of the larva depends on conditional nutritional feeding; the larva that becomes a queen is raised in cells with more food than the workers—building queen cells and supplying them with the food necessary for their development. In these species, the queen cells are normally located on the periphery (Smith *et al.* 2008; Smith and Suarez 2010).

Kerr (1950) proposed the Melipona model of caste determination, in which two unrelated *loci*, each with a pair of alleles, interact to produce the specific genotype of a queen. Only when both *loci* are in a heterozygous condition can the individual become a queen. But the expression of the queen phenotype will require an adequate quantity or quality of food (Quezada-Euán, 2018). Due to the evolutionary complexity of eusociality in insects, it is relevant to consider for its understanding in addition to a consideration of caste determination by the amount of food ingested; the modification of molecular pathways related to development, behavior, neurobiology, physiology, and morphology as well as those external routes that may affect (Figure 6) (Easter, 1978).

CONCLUSION

The biogenesis, regulation and functions of microRNA in Eukarya are still obscure, however, some light has been shed on the molecular basis of caste differentiation in eusocial bees modulated by 2 miRN transfer pathways; endogenous and exogenous.

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Compliance with ethical standards. The nature of this work did not required the approval of a bio ethical committee.

Data availability. The authors declare that all the data was retrieved from the public NCBI database and were used and processed for this manuscript as downloaded.

Author contribution statement (CRediT). D.M. Moreno Cálix – Conceptualization, writing-original draft. **F. Guillén-Chable** - Formal analysis, **M.A. Gallardo Flores** - supervision, **K.J. Cantarero** – writing – review and editing, formal analysis, **L.C. Rodríguez-Zapata** – Conceptualization, Writing-review and editing, **E. Castaño** – Writing-review and editing, visualization.

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