Universidade Estadual de Mato Grosso do Sul
Unidade Universitária de Dourados
Programa de Pós- Graduação em Recursos Naturais

INFLUÊNCIA DA TEMPERATURA NA SOBREVIVÊNCIA E COMPOSIÇÃO CUTICULAR DE TRÊS ESPÉCIES DE FORMIGAS E EFEITO ANTI-INFLAMATÓRIO DA PEÇONHA DE *Ectatomma brunneum*

Bianca Ferreira Duarte

Dourados – MS
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“Para se ter sucesso, é necessário amar de verdade o que se faz. Caso contrário, levando apenas o lado racional, você simplesmente desiste. É o que acontece com a maioria das pessoas”

(Steve Jobs)

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SUMÁRIO

RESUMO

Os insetos sociais chamam a atenção de inúmeros pesquisadores devido a sua complexa organização social. Uma das características marcantes envolvendo esta organização, é a capacidade que formigas têm de se comunicar por meio de compostos químicos que possibilitam a estes reconhecer se outro inseto pertence ou não a sua colônia. Este estudo, foi realizado a fim de identificar se os hidrocarbonetos e ácidos graxos que constituem a cutícula de formigas das espécies de *Atta sexdens, Odontomachus bauri* e *Ectatomma brunneum* eram capazes de se rearranjar quando submetidas a diferentes temperaturas. Em um segundo momento foi realizado um estudo de composição das proteínas e da ação anti-inflamatória da peçonha de *Ectatomma brunneum.* Dos resultados obtidos, foi analisado que esta capacidade de adaptação, conhecida também como plasticidade, foi atribuída somente aos hidrocarbonetos. Foi observado, também, que a espécie da formiga pode ser um fator determinante na sua habilidade adaptativa, uma vez que características como hábitos de forrageio e a localização geográfica de ninhos podem influenciar na capacidade da formiga resistir mais ou menos a determinada temperatura, por exemplo, a formiga que apresentou maior resistência a temperaturas elevadas, que foi a formiga da espécie *Ectatomma brunneum* costuma forragear em áreas de vegetação aberta e pode ser encontrada forrageando em qualquer hora do dia ou da noite. Uma análise das características estruturais dos hidrocarbonetos presentes mostrou também que estes podem influenciar nesta capacidade de resistência, uma vez que, de acordo com o aumento ou diminuição da temperatura o tamanho de cadeia e a classe dos hidrocarbonetos foram se rearranjando. De modo geral, uma maior percentagem de hidrocarbonetos lineares e hidrocarbonetos de cadeia longa foi apresentado em temperaturas abaixo de 30ºC. Dos resultados obtidos com a peçonha de *E. brunneum* , foram identificadas quatro proteínas (Dipeptidil peptidase 4; Tensin; D-glucuronil C5-epimerase e Quinase de adesão focal). Os testes realizados com camundongos da linhagem Swiss, mostraram que a peçonha reduziu o recrutamento de leucócitos para a região da inflamação, além de diminuir a formação de edema e hiperalgesia mecânica. Estes resultados apontam que a peçonha de *E. brunneum* possui efeito anti-inflamatório.

Palavras-chave: Comunicação química; Hidrocarbonetos cuticulares, Atividade anti-inflamatória.

ABSTRACT

Social insects catch the attention of countless researchers because of their complex social organization. One of the hallmarks of this organization is the ability of ants to communicate through chemical compounds that allow to recognize if another insect belongs to their colony or not. This study was carried out in order to identify if the hydrocarbons and fatty acids constituting the cuticle of ants of the species *Atta sexdens, Odontomachus bauri* e *Ectatomma brunneum* were able to rearrange when they were submitted to different temperatures. In a second moment, a study of the composition of the proteins and of the anti-inflammatory action of *Ectatomma brunneum* venom was carried out. From the results obtained, it was found that this adaptation capacity, also known as plasticity, was attributed only to hydrocarbons. It was also observed that the ant species may be a determining factor in their adaptive ability, since characteristics such as foraging habits and the geographic location of nests may influence the ant's ability to withstand more or less a certain temperature, for example, the ant that presented higher resistance to high temperatures, which was the ant of the species *Ectatomma brunneum* usually forages in areas of open vegetation and can be found foraging at any time of day or night. An analysis of the structural characteristics of the hydrocarbons present also showed that these may influence this strength capacity, since, according to the increase or decrease in temperature, the chain size and class of the hydrocarbons were rearranged. In general, a greater percentage of linear hydrocarbons and long chain hydrocarbons were present at temperatures below 30 ° C. From the results obtained with the *E. brunneum* venom, four proteins (Dipeptidil peptidase 4; Tensin; D-glucuronyl C5-epimerase and focal adhesion kinase) were identified. The test performed with Swiss strain mice showed that the venom reduced the recruitment of leukocytes to the region of the inflammation, in addition to decreasing the formation of edema and mechanical hyperalgesia. These results indicate that *E. brunneum* venom has an anti-inflammatory effect.

Keywords: Chemical communication; Cuticular hydrocarbons; Anti-inflammatory activity.

CAPITULO 1: CONSIDERAÇOES GERAIS

1.1 A ORDEM HYMENOPTERA

Os insetos pertencem ao maior e mais diversificado grupo de animais existentes na Terra, podendo ser encontrado em diversos habitats (Grimaldi et al., 2005). Os insetos sociais compreendem os cupins, as formigas e algumas espécies de abelhas e de vespas, sendo que estes três últimos fazem parte da ordem Hymenoptera (Wilson, 1971; Gullan e Cranston, 2014). Neste sentido, para que um grupo possa ser considerado eussocial, alguns pressupostos devem ser considerados, como a sobreposição das gerações, divisão de castas de trabalho entre os indivíduos inférteis que colaboram com a criação da prole do(s) indivíduo(s) fértil(eis) e, indivíduos de uma mesma colônia ajudando de forma cooperativa na criação da prole (Wilson, 1971).

1.2 A FAMILIA FORMICIDAE

Dentro da classe dos Himenópteros, a família Formicidae compreende todas as formigas, e apresenta até o momento aproximadamente 13 mil espécies e subespécies, distribuídas em 17 subfamílias (Bolton, 2018). O sucesso atribuído a sua evolução permitiu que se tornasse a espécie mais rica e dominante de todos os insetos eussociais, estando presente em toda a Terra (Brady et al., 2006; Holldobler e Wilson, 1990). Estima-se que cerca de um terço da biomassa animal da floresta amazônica seja composta de formigas e cupins (Holldobler e Wilson, 1990).

As formigas empregam as formas mais complexas de organização social, sendo a sua diversidade local substancial, excedendo em muito a de outros insetos sociais (Holldobler e Wilson, 1990). Suas espécies polimórficas possuem duas castas principais, as rainhas e as operárias. As operárias podem ser monomórficas ou dimórficas. Quando apresenta dimorfismos podem ser divididas de acordo com o seu tamanho corpóreo em operarias pequenas, médias e grandes. O polimorfismo vem acompanhado ainda do polietismo, ou seja, o papel da rainha se restringe a ovoposição enquanto as operárias cabe todos os outros trabalhos (Gullan e Cranston, 2014).

Contudo, mesmo que todas as formigas sejam eussociais, as espécies variam em seu grau de integração colonial, as sociedades menores de muitas formigas *ponerinas* por exemplo, são caracterizadas pela competição interna sobre a reprodução, enquanto as colônias maiores e

mais avançadas, como as das formigas cortadeiras, exibem uma unidade de propósito que rivaliza com alguns organismos unitários (Delabie et al., 2015). Neste contexto, a evolução social em formigas está relacionada as transições na evolução e como cada espécie é capaz de se organizar biologicamente.

.A capacidade de adaptação das formigas a diferentes microclimas se correlacionam com as suas características morfológicas. Um exemplo disto são as formigas que habitam as florestas tropicais que apresentam temperaturas mais elevadas e baixa umidade relativa, faz com que formigas arborícolas sofram maior exposição a variações climáticas do que aquelas que forrageiam sobre a sombra ou serapilheira. (Hood e Tschinkel, 1990).

A subfamília *Ecatatomminae* é composta por 266 espécies no mundo todo, com 112 ocorrendo na Região Neotropical, e 50 presentes no Brasil. Em relação a sua biologia, apresenta espécies capazes de nidificar no solo, na serapilheira, em troncos em decomposição ou mesmo no estrato arbóreo e arbustivo de florestas (Camacho e Feitosa, 2015). Destas, o gênero *Ectatomma* é o mais comumente coletado da família na Região Neotropical, sendo endêmica desta região possuindo 11 espécies conhecidas no Brasil (Kugler e Brown, 1982). São conhecidas por serem formigas grandes, abundantes e conspícuas e com preferência por habitats quentes (Camacho e Feitosa, 2015).

A subfamília *Porinae* recebe atenção por sua condição de "caçadora primitiva", apresenta cerca de 1.195 espécies em 47 genêros em todo o mundo, e destas, 15 espécies ocorrendo no Brasil. Nesta família, as operarias são parecidas entre si e há pouca diferenciação entre rainha e operarias. O gênero *Odontomachus* apresentam ninhos pouco elaborados localizados geralmente na serapilheira, madeira em decomposição, sob troncos caídos, pedras e folhas acumuladas em galhos de árvores e epífitas (Lattke, 2015).

A subfamília *Myrmicinae* constitui a maior subfamília de formigas, com mais de 6700 espécies e subespécies e 155 gêneros (AntWiki, 2018). Destas, a espécie *Atta sexdens* possui um alto grau de polietismo, sendo considerado um dos mais complexos dentre todas as formigas, suas operarias podem ser encontradas forrageando em qualquer hora do dia ou da noite, e seus ninhos podem ser encontrados em qualquer local adequado, desde o fundo do solo até os ramos superiores das árvores (Wilson, 1980; AntWiki, 2018). São conhecidas pelo hábito de cortar material vegetal fresco, sendo por isso consideradas verdadeiras pragas agrícolas (Wilson, 1980).

Das espécies descritas anteriormente, *A. sexdens* é a espécie que apresentou maior número de estudos devido a sua ampla distribuição. Há estudos que apontam a variação de seu perfil cuticular devido a fatores tais como dieta (Valadares et al., 2015), sub-castas

(Valadares e Nascimento, 2016) e por contato com outros compostos como o β-eudesmol, por exemplo (Marinho et al., 2008). *E. brunneum* teve o perfil químico cuticular de sua cutícula analisado por *Fourier transform infrared photoacoustic spectroscopy*, com o objetivo de diferenciar castas e sexo de formigas pertencentes a mesma colônia (Antonialli-Junior et al., 2008) e também diferenças relacionadas a dieta (Bernardi et al., 201). *O. bauri* ainda não apresenta informações na literatura referente a estudos envolvendo sua composição química cuticular.

O padrão de forrageio de algumas espécies de formigas depende fortemente da temperatura da região em que habitam (Cerda et al., 1998; Cros et al., 1997), fazendo com que suas diferentes habilidades influenciem o dia e a hora que elas se encontram ativas (Cros et al., 1997). Das espécies escolhidas para realizar o nosso estudo, *E. brunneum* possui hábito de forrageio diurno e noturno, em áreas de vegetação aberta (Overal, 1986), a espécie *A. sexdens* costuma limitar seus horários de forrageio em horários específicos, conforme a disponibilidade de recursos ambientais e fatores microambientais (De vanconcelos, 1990). *O. bauri*, contudo, apesar de apresentar forrageio diurno e noturno, restringe sua atividade de forrageio em ambientes sobre madeira e serapilheira, fazendo com que se submeta o mínimo possível a variações ambientais (Oliveira e Holldobler, 1989).

1.3 A IMPORTÂNCIA DOS HIDROCARBONETOS E ÁCIDOS GRAXOS ATUANDO NOS INSETOS SOCIAIS

Os insetos possuem uma superfície cerosa impermeável constituída por hidrocarbonetos (Breed e Stiller, 1992). Suas principais funções são de servir como uma camada cuticular impermeabilizante e atuar na comunicação química, já que estes hidrocarbonetos cuticulares são os principais responsáveis em servir como feromônios de reconhecimento. (Heftz, 2007; Blomquist e Bagnères, 2010).

Os hidrocarbonetos cuticulares são candidatos particularmente atrativos como sinal de reconhecimento devido a sua estabilidade química, baixa volatilidade e sua diversidade estrutural, possibilitando uma grande variabilidade na composição cuticular (Dani et al., 1996). Há relatos de centenas de hidrocarbonetos cuticulares, sendo as classes principais os alcanos lineares, alcanos ramificados e os alcenos, com comprimentos de cadeia carbônica variando de 21 a 40 carbonos, sendo que em alguns casos, há relatos de até 50 carbonos. (Blomquist e Bagnères, 2010; Ginzel e Blomquist, 2016).

Os hidrocarbonetos cuticulares compõem misturas complexase é devido a grande diversidade que se torna possível a colônia ter uma assinatura química própria (Ginzel e Blomquist, 2016). O reconhecimento dos hidrocarbonetos entre os companheiros da colônia contribui para processos como o de proteção a colônia, sendo ainda que estudos realizados mostram que a composição dos hidrocarbonetos cuticulares é especifica para espécie, sexo, castas e colônias (Breed e Stiller, 1992; Singer, 1998).

Deste modo, os hidrocarbonetos compreendem a maioria dos compostos químicos cuticulares das formigas, e compostos como os ácidos graxos, ésteres e esteróis estão presentes em menores quantidades (Stanley-Samuel et al., 1988). Destes, os ácidos graxos apresentam extrema importância na biologia de insetos devido a sua função de atuar no armazenamento de energia metabólica, na estrutura celular, na biomembrana e na fisiologia celular (Stanley-Samuel et al., 1988).

Os ácidos graxos apresentam uma composição qualitativa similar para todas as ordens de insetos, sendo os ácidos mirístico, miristoléico, palmítico, palmitoléico, esteárico, oleico, linoleico e linolênico os mais comuns (Thompson, 1973). Fatores externos são capazes de alterar a composição química dos insetos sociais, como dieta, estágio de vida, estágio reprodutivo e fatores ambientais. Estas alterações podem ocorrer devido a uma certa propriedade que estes insetos possuem de reajustar sua composição química a fim de sobreviver a alterações drásticas (Blomquist e Bagnères, 2010).

A temperatura ambiental é um dos fatores cruciais capazes de alterar o comportamento de formigas, visto que estas evitam forragear a certas temperaturas, e é por isso que possuem horários específicos de forrageamento do dia ou ano (Jayatilaka et al., 2011). Estudos que evidenciem a influência da temperatura em formigas já foram realizados como o de Bouchebti et al., 2015 que mostraram que a estrutura de ninhos podem variar de acordo com a capacidade que formigas tem de suportarem temperaturas elevadas, e estudos que evidenciam que os hidrocarbonetos cuticulares são alterados devido a mudanças na temperatura já foram estudados em trabalhos anteriores (Michelluti et al., 2017).

1.4 ATIVIDADES BIOLÓGICAS EM PEÇONHAS DE FORMIGAS

A priori, o sucesso evolucionário das formigas se deve, principalmente, à sua característica eussocial que lhes permitiu desenvolver estratégias complexas como as

secreções de peçonha, cuja função primordial é defender o ninho contra predadores, patógenos microbianos, competidores de formigas e caçar presas (Touchard et al., 2016).

Constituídos por proteínas, peptídeos, alcaloides e amidas, a peçonha de formigas pode ser considerada uma fonte promissora de novos compostos, ainda que comparado aos outros animais peçonhentos, seja pouco explorada (Touchard et al.,2014; Fontana et al., 2015). Causa esta que pode ser atribuído a pequena quantidade de material biológico que é encontrado em seu reservatório glandular, o que dificulta a identificação de seus componentes e análise de suas atividades farmacológicas (Santos et al., 2011; Fontana et al., 2015).

Neste sentido, a análise proteômica é uma ferramenta útil a fim de ajudar a desvendar a composição proteica e a atividade biológica dos constituintes de peçonhas. Identificar os componentes presentes pode auxiliar no desenvolvimento de novos compostos de interesse estratégico para as indústrias farmacêutica e química em geral, possibilitando o desenvolvimento de novos fármacos (Fontana et al., 2015).

Um exemplo disto é a formiga *Solenopsis invicta*. Estudos realizados a partir dos compostos presentes em sua peçonha relataram atividade antimicrobiana relacionada a um alcaloide (Park et al, 2008). Atividade antifúngica também foi relatada, sendo esta função por sua vez atribuída ao alcaloide piperideína isolado desta mesma espécie (Dai et al., 2011), também o alcaloide isosolenopsina A demonstrou forte inibição da isoforma neuronal do óxido nítrico-sintase, que tem efeito significativo nas reações adversas das ferroadas (Yi et al., 2003).

Além disto, a peçonha da formiga *Pachycondyla sennaarensis*, apresentou atividades antitumoral e anti-inflamatória (Badr et al., 2012; Pan e Hink, 2000). Atividades parasiticida e antibacteriana foram atribuídas ao peptídeo isolado Δ-Myrtoxin-Mp1a (Mp1a), na peçonha da formiga *Myrmecia pilosula* (Lima et al., 2016). Peptídeos foram identificados como fontes de atividades antibacteriana em estudos utilizando a peçonha da formiga *Tetramorium bicarinatum*, devido ao peptídeo isolado bicarinina (Téné et al., 2016) e na peçonha da formiga, *Pachycondyla goeldii*, devido ao peptídeo ponericin isolado de sua peçonha, vale ressaltar que neste estudo foi identificado ainda atividade inseticida na peçonha desta espécie (Orivel et al., 2001),

1.5 ATIVIDADE INFLAMATORIA E ANTI-INFLAMATÓRIA RELACIONADAS A PEÇONHAS DE FORMIGAS

Uma única ferroada de formiga é capaz de causar graves consequências, tais como dor aguda, sudorese, náuseas, alergias, choque anafilático, taquicardia, entre outros (Blum, 1958). É por isso que estudos que visam identificar os componentes de peçonhas são úteis no sentido de ajudar a compreensão de sua ação no organismo humano e auxiliar no desenvolvimento de alérgenos recombinantes para usos diagnósticos e terapêuticos (Santos et al., 2011).

Os peptídeos presentes em peçonhas de formigas podem ser classificados em dois grupos: os peptídeos citolíticos e os peptídeos neurotóxicos. Os peptídeos citolíticos são constituídos de pequenos peptídeos que vêm acompanhado frequentemente de atividades inseticida, hemolítica e/ou atividade antimicrobiana, enquanto que os peptídeos neurotóxicos são empregados com o objetivo de auxiliar na rápida imobilização da presa, que agem através de neurotoxinas paralisantes (Touchard et al., 2016).

Em relação ao estudo de proteômica, as proteínas podem ser classificadas em: proteínas tóxicas associadas à difusão da peçonha e toxicidade a presas ou predadores; proteínas neurotóxicas; proteínas que promovem a difusão da peçonha ou modulam os mecanismos de defesa das vítimas, proteínas que promovem danos nos tecidos ou causam inflamação; proteínas alérgenas; proteínas antimicrobianas e as proteínas de função desconhecida (Touchard et al., 2016).

Contudo, é possível encontrar também estudos que evidenciam o uso de peçonhas de Hynmenopteros no sentido de servir como fontes terapêuticas vinculadas ao alívio da dor e tratamentos anti-inflamatórios. Um exemplo disso, é a peçonha da formiga *Pseudomyrmex triplarinus* que demostrou alívio de dor e inflamação de pacientes com artrite reumatoide (Altman et al., 1984), para a qual foi posteriormente comprovada a atividade anti-inflamatória através do modelo de edema animal induzido por carregenina (Pan e Hink, 2000). A atividade anti-inflamatória desta peçonha está associada a um complexo multiproteico de seis myrmexinas, designadas myrmexinas I – VI (Pan e Hink, 2000). Além disse, em abelhas, a peçonha *Appis melífera* foi capaz de suprimir a inflamação artrítica em ratos, quando estes foram induzidos com adjuvante completo de Freund (CFA) (Kang et al., 2002).

1.6 OBJETIVO

1.6.1 OBJETIVO GERAL

Avaliar a influência da temperatura no perfil cuticular de três diferentes espécies de formigas e o potencial anti-inflamatório de *Ectatomma brunneum*

1.6.2 OBJETIVOS ESPECÍFICOS

Comparar o perfil químico cuticular de três diferentes espécies de formigas contidas em uma região subtropical no Brasil, situada no Estado de Mato Grosso do Sul.

Avaliar se diferenças nos hidrocarbonetos cuticulares e ácidos graxos podem ser responsáveis pela maior ou menor resistência destas formigas a variações de temperatura.

Realizar ensaio anti-inflamatório em camundongos com a peçonha bruta de *Ectatomma brunneum* e correlacionar com a sua composição química.

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CAPITULO 2: Effect of temperature on survival and cuticular composition of three different ant species

Introduction

Subtropical organisms are exposed to different seasonal variations, and to cope with such changes, they can acclimate themselves though changes in physiology, morphology and/or behavior (Angilletta et al., 2009). Ants, in particular, use cuticular hydrocarbons (CHCs) present on their body surface to respond to these variations. In other words, these insects can adjust the content of cuticular chemical compounds to act as a barrier against desiccation (Menzel et al., 2017).

CHCs are specifically found in the outermost layer of the cuticle (Gullan and Cranston, 2007). This structure is coated with chemical compounds that have, above all, two primordial functions. The first is to perform waterproofing, and the second is to perform chemical communication (Blomquist and Bagnères, 2010). In addition to the hydrocarbons, Lockey (1988) also highlights other compounds resident in the insect cuticle, including fatty acids, esters, alcohols, acylglycerides, phospholipids and glycolipids. In addition, Stanley-Samuelson et al. (1988) also report palmitic acid, stearic acid and monounsaturated oleic acid as standard fatty acids in insects in general (Stanley-Samuelson et al., 1988). Lipids can also act as a barrier to prevent water loss across the animal's surface (Gibbs & Pomonis, 1995). Thus, melting temperature directly correlates with the ability of an insect to maintain its water balance, i.e., allows them to withstand high temperatures (Edney, 2012).

The ability to respond to variations in temperature depends on the exercise of different adaptations, whether behavioral, morphological or physiological (Boulay et al., 2017). Qualitative and quantitative variations of certain classes of chemical cuticular compounds, as well as different chain lengths, may also determine the ability of an insect to respond to environmental changes, such as at different temperatures (Gibbs et al., 1997; Hefetz, 2007; Menzel et al., 2017; Michelutti et al., 2018). The linear alkanes, for example, act mainly to waterproof the cuticle (Gibbs, 1998; Hefetz, 2007), while branched alkanes and alkenes mediate communication (Hefetz, 2007; Lorenzi et al., 1997). Thus, straight chain alkanes have higher melting temperatures than branched alkanes of comparable carbon chain length: the higher the branching, the lower the melting temperature (Hefetz, 2007). However, Menzel et al. (2017) report that linear alkanes and methyl alkanes are important waterproofing compounds.

Both compound class and length of carbon chain are important factors that affect waterproofing the cuticle (Gibbs and Pomonis, 1995; Gibbs et al., 1997; Gibbs 1998). Menzel et al. (2018) studied the effect of temperature on the chain length of linear alkanes in the cuticles of the ants *Temnothorax longispinosus* and *Temnothorax ambiguus* and detected a strong correlation between chain length and increasing temperature. Michelutti et al. (2018) reported on the effect(s) of temperature variation on the CHCs of three wasp species and identified a significant change in compounds with longer chain length, thus supporting these compounds as "first responders" to environmental changes.

Different species and individuals within species possess specific lipid compositions, making each lipid layer unique. This means that different individuals exhibit more or less water loss at a given temperature, in turn, resulting in a specific critical temperature (Gibbs, 1998). In general, the composition of fatty acids is similar for all insects, and quantitative differences are more related to differences in taxonomic groups (Thompson, 1973). In addition, these fatty acid-related differences may still be affected by factors, such as order, family, species, flight activity, life stage, reproductive stage, as well as environmental factors that include photoperiod, temperature, and diet (Rosumek et al., 2017; Stanley-Samuelson et al., 1988; Thompson, 1973). Studying *Formica fusca* and *Myrmica rubra* ants, Rosumek et al. (2017) found that individual fatty acid profiles change as a function of diet and that these changes become more pronounced over time. In addition, the fatty acids appear to be involved in the formation of CHCs, as highlighted by Blomquist and Bagnères (2010).

Odontomachus bauri lives especially in forest areas; therefore, they use treetops for orientation in foraging since this represents cover with particularly restrictive lighting conditions characteristic of tropical forests (Oliveira & Holldobler, 1989). Foraging activity occurs during the day and at night in nests and under the wood and serrapilhiera, exposing them to the little variation of climatic conditions (Ehmer & Holldobler, 1996). *Ectatomma brunneum* nests in the soil and is abundant in areas of open vegetation or altered environments, either by anthropic or natural activity, close to human habitations, such as plantations, grasses, grasslands, roads and forest clearings (Overal, 1986; Vasconcelos, 1999). This species forages during the day and night avoiding only the hottest times of the day (Overal, 1986). The foraging activity of *Atta sexdens* is limited by the availability of resources and microenvironmental factors. During the drier months, foraging shifts to a sharp nocturnal pattern, and in the summer, foraging may also change to nighttime to avoid extremely high temperatures.

Based on the foraging behavior of ant species detailed above, this study hypothesizes that different ant species respond to temperature changes in different ways and that such differences may be associated with cuticle hydrocarbons (CHCs) and fatty acids. As model ant species, *A. sexdens, O. bauri* and *E. brunneum* were used for experimental analyses.

Material and methods

Collection and acquisition of the ants

Ants of three species were collected in the municipality of Dourados (22° 13'24.39 "S; 54° 54'44.53" W) and Ponta Porã (22° 32'10 "S; 55° 43'32 " W), in the state of Mato Grosso do Sul - MS, Brazil, in December 2017. In addition, since the composition of CHCs in these ants may vary according to the ant function within their colony, only foragers (exterior workers) were collected. These foragers are most affected by temperature variation in their environment (Menzel et al., 2018; Wagner et al., 2001).

Collections were carried out during early morning and later afternoon since these are the best times to find foraging ants. For each species, approximately 300 ants were collected and divided into 7 subgroups containing 30 workers each for experiments, including a control group of 10 ants.

After collection, control ants were weighed and immediately frozen for extraction of cuticular compounds, thus avoiding any effects of experimental manipulation. Foragers collected for temperature experiments were taken to the laboratory where they were acclimated for a maximum period of 7 days under a constant temperature of 26ºC, making use of an air conditioner. A thermometer was used to monitor the ants, and the mean temperature was that recorded for the month prior to the experiment. They were kept in plastic pots of 500 mL containing water and honey in Eppendorf tubes as a food resource.

According to Zavatini (1992), Mato Grosso do Sul state has two main annual seasons, one hot and humid and the other cold and dry. The temperatures selected for the study were chosen according to the average temperature of the two main weather observation station (Empresa Brasileira de Pesquisa Agropecuária–Embrapa/Oeste) for the City of Dourados-MS during 2017. Our model ants are well acclimated to these temperature shifts. The cold and dry season presented a mean temperature of 21.6°C±4.1 with a minimum temperature of 16.4°C±4.5. The hot and humid season had an average temperature of 25.3°C±2.4 with an average maximum of 32.0°C±3.4 Thus, the experimental temperatures were selected about

five degrees up and down from the average minimum and maximum, respectively, for that year.

Temperature variation experiments

The experiment was carried out following the model described by Bouchebti et al. (2015) with modifications. Ants were individually inserted into an Eppendorf tube containing a piece of moist cotton attached to its cap to humidify the air. For each temperature tested, a group of 30 foragers of each species were submitted to a digital ultrathermal water bath for 5 hours. The tubes containing the ants were placed in the temperature maintenance system under different temperatures for five hours. The temperatures tested for the experiment were 10°C, 15°C, 20°C, 25°C, 30°C, 35°C and 40°C.

In accordance with Bouchebti et al. (2015), the ants were monitored every 30 minutes in order to monitor their survival, and the ants that did not support this time interval were removed. The experiment lasted a total of 5 hours. As a control after collection, ten individuals of each species were sacrificed by freezing, followed by chemical analyses. Following chemical assessment, all ants of the three species submitted to the temperature experiment were removed from the temperature maintenance system, and those that were not dead were sacrificed by freezing for 10 minutes for further extraction of the cuticular compounds of ten ants from each treatment. Ants, ants that died and survived to the end of the experiment, were submitted to chemical analyses of cuticular compounds. Control ants were sacrificed by freezing, as described for the other samples.

The CHCs of 10 foragers were extracted from each exposure group at each temperature and control. Each ant was then dipped in a glass vessel containing 2 mL hexane (Tedia, HPLC grade) for a period of 3 minutes. Flasks containing extract were dried at room temperature and then cooled (-4°C) for a maximum of 7 days for further solubilization and analysis using gas chromatography coupled to mass spectrometry (GC-MS). After extraction of CHCs extraction, all foragers were subjected to extraction of their lipid contents.

After analyzing the hydrocarbon content of ants, they were then subjected to lipid extraction. Lipid extraction was performed following the methodology described by Bazazi et al. (2016) with modifications. Ants were inserted into glass vials, and 2 mL of chloroform (synth 100% m/m) were added to each flask. After 24 hours, this solvent was removed, and 2 mL of chloroform were added again. This procedure was performed once again for a total of three extractions. After extraction and drying of the chloroform, each sample was esterified by adding 2 mL of hexane and 2 mL of potassium hydroxide (85% m/v) in methanol (Vetec

99.8% w/w) 2 mol/L to the sample. This mixture was vortexed for 5 minutes, after which the two-phase separation could be observed. The upper phase consisted of the solvent composed of the esters of fatty acids, and the lower phase was aqueous. The upper phase, which was used in the analysis of lipid compounds, was dried in an exhaust hood and stored for analysis using GC-MS.

Chromatographic analysis

Analysis of the hexane fraction

For analysis using gas chromatography coupled to mass spectrometry (GC-MS), the dried samples were solubilized by vortexing in 200 μL of hexane and then transferred to vials. The samples were analyzed using a gas chromatograph (GC-2010 Plus, Shimadzu, Kyoto, Japan) coupled to a mass spectrometer (GC-MS Ultra 2010, Shimadzu, Kyoto, Japan) using a DB-5 fused silica capillary (J and W, Folsom, California, USA) with 5% of phenyl dimethylpolysiloxane on capillary fused silica (30 m long x 0.25 mm internal diameter x 0.25 μm film thickness). The conditions of analysis were heating ramp with initial temperature of 150ºC, reaching 280°C at 3ºC/min and remaining at the final temperature for 10 minutes. Helium (99.999%) was used as drag gas (1 mL/min), and injections were 1 μL in splitless mode. The injector, detector and transfer line temperatures were 250°C, 250°C and 290°C, respectively. Scanning parameters of the mass spectrometer included electron beam ionization voltage of 70 eV, with m / z 40 to 600 and scanning range of 0.3 s.

Compound identifications were performed using the calculated retention index (IRC) (Van den Dool and Kratz, 1963) and the linear alkane (C_7-C_{40}, Sigma Aldrich purity ≥ 98%) standard, along with comparisons of the IRC with indexes found in the literature (Bonavita-cougourdan et al., 1991; Brophy et al., 1983; Michelutti et al., 2017; Moore et al., 2014; Smith et al., 2016; Yusuf et al., 2010), associated with interpretation of mass spectra obtained from the samples and compared with the databases (NIST21 and WILEY229). Compounds containing less than 0.1% were not shown in the tables. Major compounds were those that represented at least 3% of the total relative percentage area of the CHCs and 10% of the fatty acids. For comparison of characteristic CHCs of each species, analyses of CHCs from the control group were performed. Control foragers were those ants without any experimental manipulation.

Analysis of the chloroform fraction

Samples extracted with chloroform and esterified were solubilized by adding 500 µl of hexane and analyzed using gas chromatograph coupled with mass detector (GC-MS Ultra 2010, Shimadzu Kyoto Japan). Chromatographic separation was performed on a fused silica column RTx-5MS (5% Phenyl- 95% Polydimethylsiloxane; 30 m x 0.25 mm ID, 0.25 µm) (Restek, Bellefonte, PA, USA). Helium gas (purity 99.995%) was used as the carrier at a constant flow rate of 5 mL/min. The temperature was set to start at 130°C and was maintained for 1 minute when it was raised to 170°C at 6.5°C/minute. Subsequently, another elevation of 170°C to 215°C was performed at 2.75°C/min, and the temperature was maintained for 12 minutes. Finally, a final elevation was performed from 215°C to 230°C at 40°C/minute. The temperatures of the injector, interface, and detector were 270, 280 and 280ºC, respectively. The 1 µL samples were injected in Split mode (1:20). Identification of the fatty acid methyl esters was performed by comparison with the fatty acid retention times of standards obtained from Sigma-Aldrich (dodecanoic acid ≥98%, myristic acid> 99%, palmitic acid> 99%, palmitolytic acid> 98.5%, stearic acid ≥98.5% and oleic acid> 99%) eluted under the same conditions.

Statistical analysis

From the spreadsheets obtained in the analysis of the chemical compounds extracted at each temperature, a nonparametric permutation analysis (PERMANOVA) was used to test the existence of significant differences among the temperatures. Euclidean distance was used to construct the similarity matrix, and significance of comparisons was calculated from the randomization of the original matrix (9999 permutations). Only area values greater than 1% were considered, and Berferroni correction was applied. All analyses were performed using the R platform (R Core Team, 2017).

Results

Thermotolerance

Figure 1 shows that ants of all three species survived at temperatures of 10°C to 30°C. From 35°C, 86.67% of *A. sexdens* workers and 96.67% of *O. bauri* workers survived during the experiment, while all *E. brunneum* workers survived. At 35ºC, a decrease in the number of survivors began to occur for *O. bauri* and *A. sexdens*. At 40°C, 10% of *A. sexdens* workers and 56.67% of *E. brunneum* workers survived during the experiment. At 40 ºC, a decrease in

the number of survivors for all three species was observed, During the 100-minute experiment, 100% of *O. bauri's* foragers had already died.

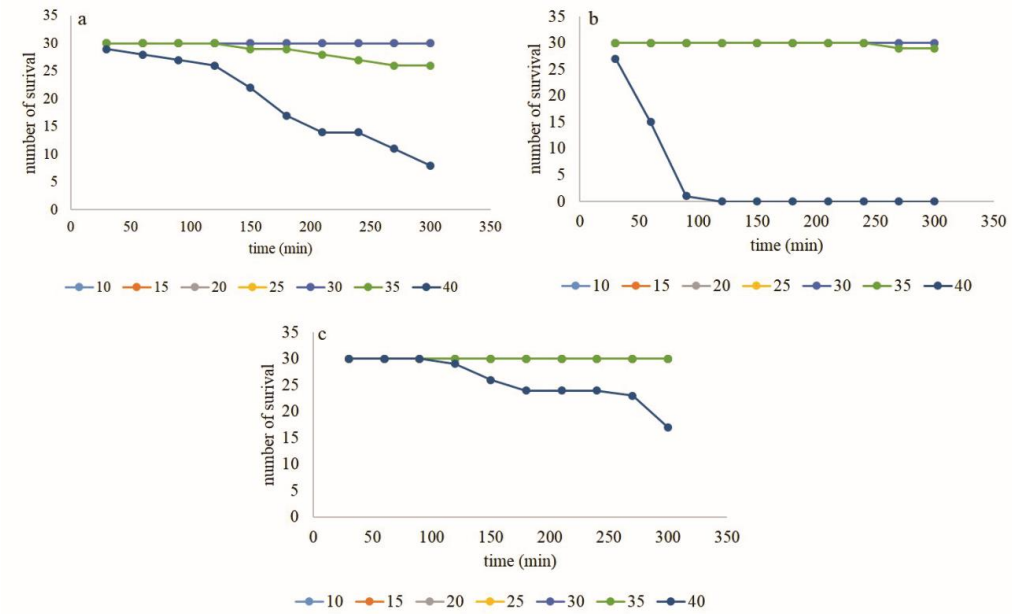

Figure 1. Probability of survival of workers of 3 species of ants under different temperature.

Analysis of cuticular chemical composition: hydrocarbons

A total of 24, 22 and 23 peaks were detected in the cuticle samples of *A. sexdens* (Table 1), *O. bauri* (Table 2) and *E. brunneum* (Table 3) samples, respectively. Compounds consisted of linear alkanes, branched alkanes and alkenes, with carbon chains varying from C_{15} to C_{32}. The Permanova analysis showed significant differences in the cuticular compounds of the three species of ants submitted to the different temperatures (Supplementary Table 1-3). Number of compounds, relative percentage area of the three different classes of compounds, and relative percentage area according to chain size were calculated, and the values are respectively presented in Figures 2, 3 and 4.

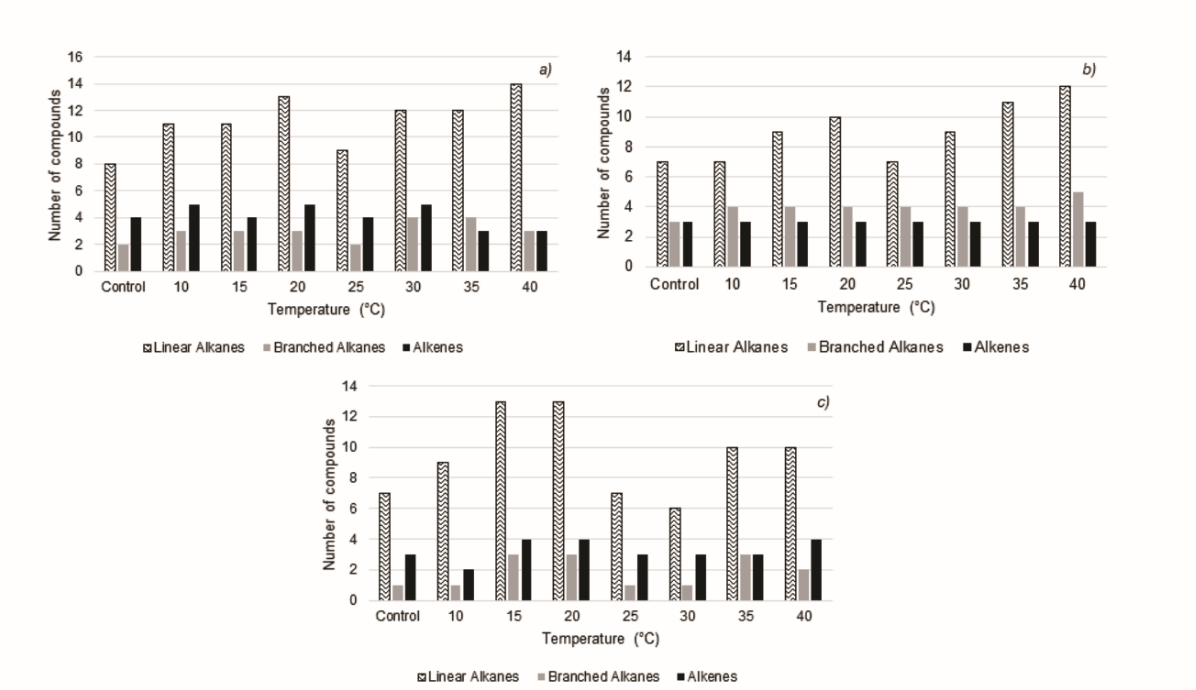

Figure 2. Number of compounds in compound classes present in the cuticle of a) *Atta sexdens*, b) *Odontomachus bauri* and c) *Ectatomma brunneum* foragers exposed to different temperatures and control.

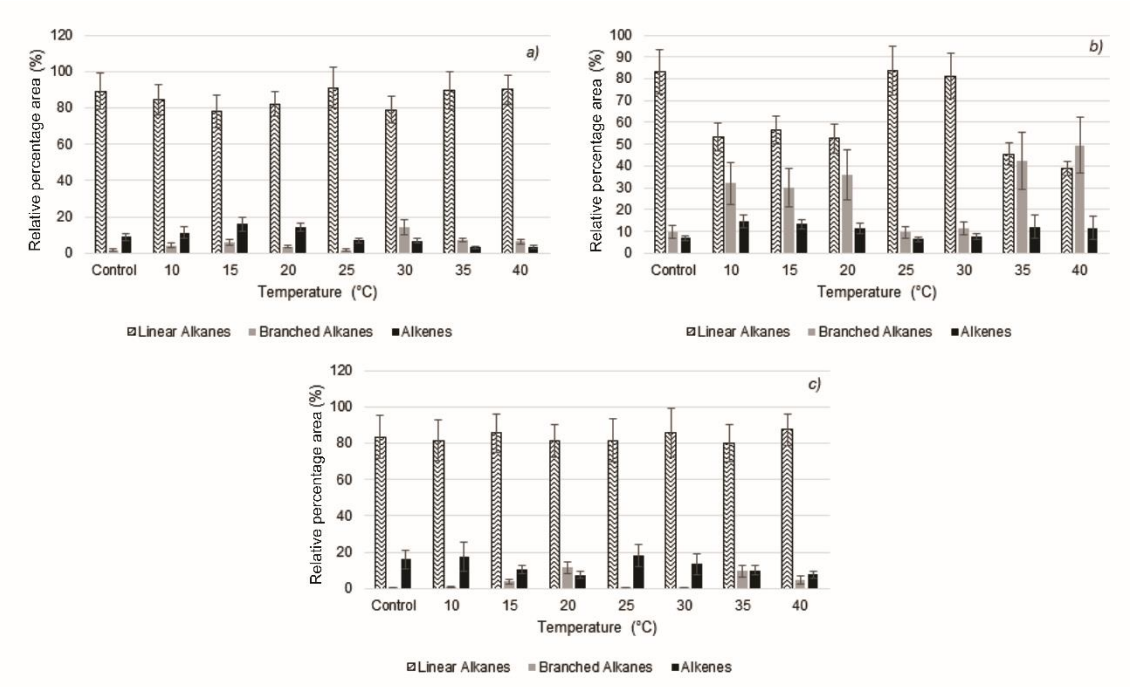

Figure 3. Relative percentage area of the three compound classes present in the cuticle of a) *Atta sexdens*, b) *Odontomachus bauri* and c) *Ectatomma brunneum* foragers exposed to different temperatures and control.

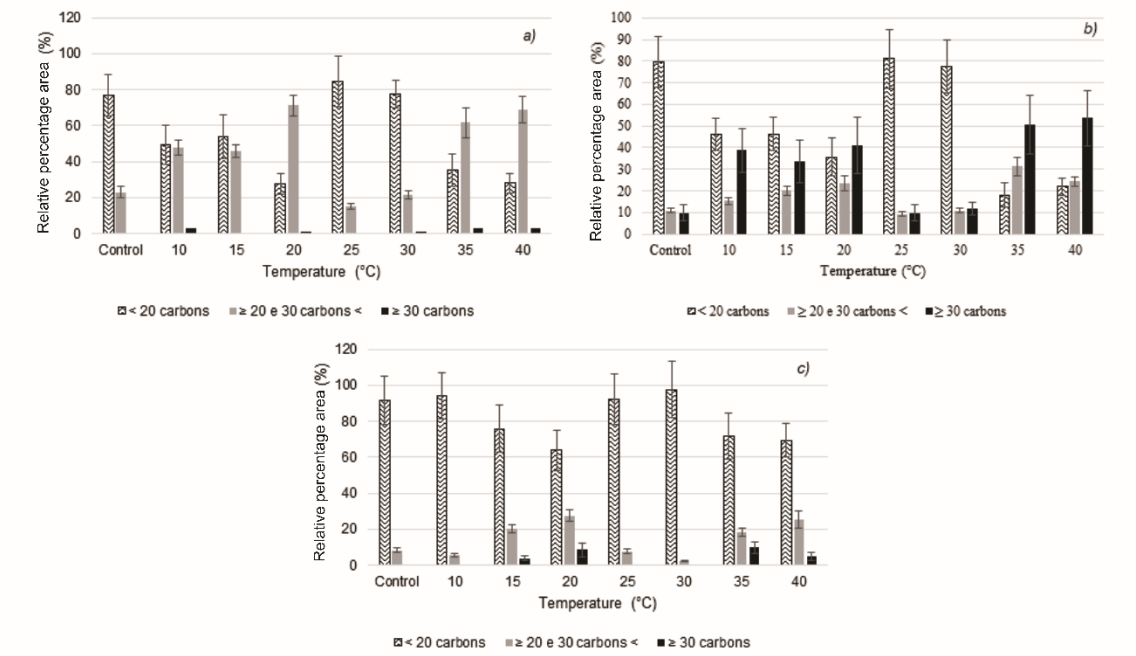

Figure 4. Relative percentage area of the compounds present in the cuticle of a) *Atta sexdens*, b) *Odontomachus bauri* and c) *Ectatomma brunneum*, according to chain size, upon exposure to different temperatures and control.

Analysis of cuticular chemical composition: fatty acids

Independent of exposure to different temperatures, six different fatty acids were identified in the samples of the three ant species studied, including lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid and oleic acid. Their relative percentage areas in the samples submitted to the different treatments and control are found in Tables 4-6. PERMANOVA analysis did not present statistical difference in the fatty acids, either in samples submitted to treatments or control, and the results are as follows: *A. sexdens*, F= 0.2308 and P= 0.9984; *O. bauri*, F= 0.3995 and P= 0.9855; and *E. brunneum*, F= 0.5978 and P= 0.7786.

Discussion

The CHCs identified in the cuticle of the thre ant species (Tables 1-3) included the linear alkanes, branched alkanes and alkenes. Studies in the literature also confirm that these are the main classes of CHCs typically identified in the cuticle of insects (Blomquist and Begnères, 2010; Howard and Blomquist, 2005; Martin et al., 2004; Zhu et al., 2006). Linear alkanes had

a higher number and percentage of compounds among the three species (Figures 2 and 3). Since we studied only worker/foragers, the linear alkanes with longer chains may be linked waterproofing to protect from desiccation (Gibbs, 1998; Menzel et al. 2017; Wagner et al., 2001) since workers are the most exposed to the elements. In number of compounds, the second most representative class in the *A. sexdens* and *E. brunneum* samples was the alkenes and, finally, the branched alkanes, whereas in the *O. bauri* samples, branched alkanes was the most representative class. These variations in CHCs are inherent to species (Akino et al., 2002; Martin et al., 2008); therefore, CHCs function as a complementary taxonomic tool addition to species-specific variation (Kather and Martin, 2012), and they can be affected by environmental and genetic factors (Blomquist and Bagnères, 2010; Hefetz et al., 2007; Wagner et al., 2001).

When temperatures rose above 35ºC, a temperature when *A. sexdens* workers began to die, we can see an increase in the production of linear alkanes in an attempt to meet the need for waterproofing. Linear alkanes have a higher waterproofing capacity since these compounds increase the viscosity of the cuticle (Gibbs 1998; Hefetz 2007; Menzel et al., 2017; Wagner et al., 2001). At temperatures above 35°C, the alkenes suffered a reduction in their percentage area, while the branched alkanes showed an increase relative to the control group (Figure 3). However, these branched alkanes and alkenes increase the fluidity of the cuticle (Menzel et al., 2007), and as a result, these compounds are important for the communication (Gibbs, 1998; Hefetz, 2007; Le Conte and Hefetz, 2008; Lorenzi et al., 1997). However, branched alkanes and alkenes also have a lower melting point than their respective linear alkanes (Gibbs and Pomonis, 1995); therefore, it would not redound to the benefit of the ant to increase the percentage of this class of compounds when exposed to temperature variation, especially at relatively higher temperatures.

Table 2 shows that one of the major compounds of *O. bauri* is a dimethyl alkane (12,16-, 13,17-, 14,18- Dimethyldotriacontane). This feature does not benefit insects in the sense of avoiding waterproofing at high temperatures since, according to Gibbs and Pomonis (1995), the insertion of branches and instaurations decrease the melting temperature of the compounds, which may, in turn, contribute to the lower tolerance of this species at temperatures above 30ºC.

At temperatures below 25°C, branched alkanes and alkenes increased for *A. sexdens* and *O. bauri*. The increase of branched alkanes and alkenes at low temperatures is expected since these compounds increase the fluidity of the cuticle which is necessary, especially under low temperatures, for the homogenization and transmission of the compounds throughout the

cuticle (Menzel et al., 2017). In *E. brunneum*, this behavior was very similar, except at 10ºC when the number of alkenes decreased.

At temperatures of 35 and 40 ºC, branched alkanes increased in the relative percentage area of *E. brunneum* in relation to control (Figure 3). Although branched alkanes are considered to be important in mediating communication, all these compounds may actually perform dual functions, i.e., waterproofing and communicating (Boulay et al., 2017; Chown et al., 2011; Chung and Carroll, 2015; Gibbs, 2007). Menzel et al. (2007), in fact, also suggest that branched alkanes provide such dual functions.

Intermediate chain compounds increased in relation to relative percentage area and length of the carbonic chain when exposed to temperatures below 25ºC. At temperatures above 30°C, intermediate and long-chain compounds increased, while short-chain compounds were reduced relative to control. These results were expected since the melting temperature of compounds increases with increasing chain length (Gibbs and Pomonis, 1995). Long-chain CHCs tend to fuse only at higher temperatures; therefore, as the melting temperature of a compound increases, the loss of water through the cuticle decreases (Gibbs et al., 1997; Gibbs, 1998). In fact, Gibbs et al. (1997) have identified the presence of CHCs with longer chain lengths in *Drosophila melanogaster* flies under desiccation conditions. In a study with the ants *T. longispinosus* and *T. ambiguus*, Menzel et al. (2018) identified significant differences in chain length of linear alkanes in foragers which perform tasks outside the colony and similarity between nurses and queens, who perform tasks within the colony. The authors then suggested that the determining factor of cuticular chemical composition would be the climatic physical factors to which these castes and subcastes are subjected. This relationship between temperature and chain length variation of cuticular compounds was also evaluated by Michelutti et al. (2018) in three species of social wasps.

Still comparing the CHC of the ants exposed to temperature variation relative to control ants, it was possible to evaluate differences in the number and relative percentage of compounds. These results add further support to the affirmation that ants can respond to temperature variation by adjustment of CHCs, as previously reported by Gibbs et al. (1997) *with D. melanogaster*, Menzel et al. (2018) with *T. longispinosus* and *T. ambiguous,* and Michelutti et al. (2018) with the wasps *Polybia paulista, Polybia ignobilis* and *Polybia versicolor*. In addition, a detailed review of temperature-dependent CHC variation in different insect species can be seen in Otte et al. (2018).

Survival tests showed that ants exposed to a temperature of 35°C began to die, whereas in analyses of the number of compounds, relative percentage area, and chain length showed that

greater differentiation in structural properties of the compounds began to occur at this temperature. Thus, it is possible to affirm that such extreme temperature thresholds militate against the well-being of foraging ants since it damages cuticular composition in response to the temperature increase. On the other hand, Ramsay (1935), studying the cockroach *Periplaneta americana*, reported a marked increase water loss at temperatures above 30°C. Wigglesworth (1945) found similar results in a study of *Blattella germanica*. These authors highlight the conformational change of the compounds that causes the rupture of the cuticle and causes water loss. Thus, a cause of structural cuticular change in temperatures above 30°C can be attributed to the attempt of ants to match, i.e., balance, the composition of chemical constituents of their cuticle to the outside environment in order to avoid water loss.

From these results, we learn that ants tend to forage at temperatures that will result in the lowest expenditure of energy and that such temperature will, in turn, affect the foraging behavior of ants (Robinson and Fowler, 1982; Nielsen, 1986; Holldobler and Wilson, 1990). This explains why ants will always seek a range ideal for foraging. Accordingly, Lima and Antonialli-Junior (2013) evaluated *Ectatomma vizottoi* foragers and found that they are active in temperatures ranging from 14ºC to 32ºC, showing that temperature is a crucial factor in foraging behavior in this species.

Thus, quite in line with the hypothesis driving the present work, different ant species exert different strategies in response to changes in environmental temperature (Bouchebti et al., 2015; Boulay et al., 2017), and changes in the conformation of CHCs demonstrate this (Gibbs et al., 1997; Menzel et al., 2017, 2018, Michelutti et al., 2018; Wagner et al., 2001). In addition, Menzel et al. (2018) showed that the survival of a species with changing climatic conditions will depend less on its current profile and more on the phenotypic plasticity of the CHC profile. In this sense, Otte et al. (2018) emphasize that it is necessary that the insect have the capacity to express phenotypic response to changes in the environment and that such response be fast enough to accompany these environmental changes.

On the other hand, the ecological habits of different species can affect the capacity of hydrocarbons to respond to temperature changes and, consequently, the ability of ants to survive. For instance, *O. bauri* presented the least tolerance to temperature change in the survival test. Foragers of this species tend to forage in places protected from wide temperature swings, such as wood and serrapilheira, as also reported by Ehmer and Holldobler (1996), *A. sexdens*, an ant species that forages according to the availability of resources within particular microenvironments (Fowler and Robinson, 1979), presented an intermediate tolerance level to temperature variation compared to the other two species. *E. brunneum* was the most tolerant

and *de facto* forages in open field areas during both day and night. It also presented the best results in the survival test (Overal, 1986). The tolerance level of the three species also seems to reflect an ability to respond to environmental changes by altering the chemical composition of their cuticles (Fig. 1 and Tables 1-3), which is likely also a reflection of their different foraging habits.

From this perspective, the chemical compound constituents of the insect's cuticle must remain in constant "balance" in order to maintain control over body temperature and communicate with nestmates (Blomquist and Bagnères, 2010), and it is well known that temperature variations and relative humidity may affect the cuticular chemical composition of insects (Hefetz, 2007; Wagner et al., 2001).

Six fatty acids were identified in the samples of the three species. These acids exhibited neither qualitative nor quantitative differences among the species, and the major acids for the three species were palmitic and oleic. Comparing the composition of fatty acids of seven orders of insects, including Hymenoptera, Thompson (1973) detected quantitative difference between insects, and oleic and palmitoleic acids were the most frequently identified fatty acids in Hymenoptera.

The similarity of composition found in the fatty acids of the three species of ants studied agrees with the findings of Lok et al. (1975) who reported on adult ants of the species *Solenopsis invicta* and *Solenopsis richteri* and demonstrated that the composition of fatty acids of adults was similar between the two species. Rosumek et al. (2017) also found similarity in the fatty acids present in the foragers of ants of the species *F. fusca* and *M. rubra*.

Palmitic acid has already been identified in the nest material of two species of wasps, *Polistes annularis* (Espelie and Hermann, 1990) and *Mischocyttarus consimilis* (Michelutti et al., 2017); stearic acid was also identified in the nest of *P. annularis* (Espelie and Hermann, 1990). Up to now, it was believed that these acids could act as an ant-repellent substance (Espelie and Hermann, 1990; Michelutti et al., 2017); however, since these compounds are found in three species of ants, perhaps the function of these compounds is also related to the maintenance of the waterproofing structure.

Conclusion

Based on our results, it can be concluded that the forager ants studied respond differently to temperature variation. Across species, we saw the increase of compounds with longer chains and an abundance of linear alkanes, results that could be expected based on reports in the literature showing how important these compounds are for waterproofing. Changes in the conformation of CHCs are in line with the ecological characteristics of the different species because, as reported, they vary in terms of diurnal/nocturnal foraging and types of environments foraged. Thus, among the three species, *E. brunneum* foragers were more active under adverse conditions and more tolerant to temperature variation with the correspondingly appropriate changes in CHCs composition.

Tables

Table 1. Relative percentage area of compounds of *Atta sexdens* cuticle for samples exposed to different treatments and control.

		Control	**10ºC**	**15ºC**	**20ºC**	**25ºC**	**30ºC**	**35ºC**	**40ºC**
Compound	**ECL**	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD
Pentadecane[a]	1500	9.93±0.61	29.75±10.40	30.31±4.85	14.61±6.58	10.58±3.82	13.64±5.85	24.00±5.31	13.84±3.74
Hexadecane	1600	15.56±2.18 [a]	9.90±3.40 [a]	2.09±0.92	0.81±1.42	15.65±3.76 [a]	14.65±3.15[a]	0.69±1.95	1.31±2.53
Heptadecane[a]	1700	10.66±0.38	7.53±5.28	16.73±3.65	8.75±4.23	12.25±3.21	13.12±5.83	9.48±4.24	9.05±2.78
5-Methylheptadecane	1750	-	-	-	-	-	9.32±8.39[a]	0.70±2.21	-
Octadecane	1800	33.78±1.30[a]	-	-	-	40.68±6.33[a]	23.0±7.28[a]	-	2.96±9.36
9-Nonadecene	1874	3.77±0.20[a]	0.21±0.35	-	0.62±0.47	2.60±1.80	0.96±1.24	-	-
Nonadecane	1900	2.57±0.17	1.38±2.22	2.98±5.86	2.49±2.21	2.50±0.24	2.01±0.67	0.21±0.46	0.70±1.10
1-Eicosene	1994	3.30±0.13[a]	2.88±4.64	7.70±1.30[a]	3.99±2.89[a]	3.00±1.69[a]	3.40±0.73[a]	0.66±1.08	1.99±2.32
Eicosane	2000	1.12±0.08	3.82±2.69[a]	4.18±1.63[a]	1.93±1.32	0.91±0.68	0.87±0.47	-	0.63±1.03
Heneicosane	2100	-	0.67±0.66	0.52±0.31	0.80±0.16	0.10±0.01	0.15±0.11	0.47±0.26	0.51±0.52
11-Methylheneicosane	2136	1.10±0.08	0.40±0.63	3.56±4.35[a]	1.19±0.90	1.13±0.12	3.13±0.58[a]	2.16±2.66	2.00±0.64
1-Docosene	2195	1.15±0.05	7.21±4.60[a]	6.93±1.07[a]	3.78±0.97[a]	0.84±0.53	1.40±0.28	0.85±1.07	1.32±0.95
11-Tricosene	2273	0.64±0.03	0.20±0.64	0.34±0.56	5.50±4.18[a]	0.61±0.46	0.77±1.00	1.64±3.48	-
Tricosane	2300	-	1.71±0.70	0.55±0.27	1.72±0.15	0.06±0.13	0.28±0.51	1.62±0.61	1.69±0.44
11-Methyltricosane	2333	0.71±0.09	0.83±0.58	1.74±0.14	1.18±0.38	0.65±0.07	0.81±0.41	1.23±0.20	1.42±0.36
1-Tetracosene	2396	-	0.78±0.45	0.65±0.19	0.42±0.24	-	0.09±0.07	-	0.04±0.09
Tetracosane	2400	-	3.39±2.17[a]	1.06±0.11	15.34±2.16[a]	-	0.01±0.01	1.44±1.73	17.15±5.13[a]
Pentacosane[a]	2500	13.12±1.88	15.76±5.23	12.64±5.27	19.95±6.07	6.36±2.13	7.94±1.39	31.69±6.74	25.23±8.83
Hexacosane	2600	-	1.02±0.36	0.49±0.39	2.80±1.18	-	0.10±1.11	2.82±2.58	3.45±0.96[a]
Heptacosane	2700	1.71±0.12	8.52±3.76[a]	4.29±0.89[a]	9.53±2.00[a]	1.44±0.54	2.45±0.60	14.87±3.62[a]	11.09±3.88[a]
Octacosane	2800	-	-	-	1.05±0.11	-	-	0.85±1.28	1.13±0.38
Nonacosane	2900	-	-	-	1.59±0.35	-	-	0.99±1.31	0.60±0.69
x.y-Dimethylltriacontane	3060	-	3.02±0.67[a]	0.34±0.76	1.13±0.84	-	0.88±0.66	3.03±1.60[a]	3.13±1.87[a]

NI	1906	0.88±0.05	0.98±0.91	2.87±0.53	0.78±0.61	0.64±0.45	1.06±0.41	0.58±0.79	0.74±0.92
Percentage of identified compounds:		***99.12***	***99.02***	***97.13***	***99.22***	***99.46***	***99.04***	***98.41***	***99.26***

Control not subjected to temperature change. ECL= Equivalent Chain Length.

[a]major compounds for different temperatures

NI: Not identified

n=10

Table 2. Relative percentage area of compounds of *Odontomachus bauri* cuticle for samples exposed to different treatments and control.

Control. not subjected to temperature change. ECL=Equivalent Chain Length.

[a]major compounds for different temperatures.

		Control	**10ºC**	**15ºC**	**20ºC**	**25ºC**	**30ºC**	**35ºC**	**40ºC**
Compound	**ECL**	M±DP	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD
Pentadecane[a]	1500	14.18±1.60	17.14±16.19	21.21±8.23	20.79±11.21	11.78±4.08	13.5±10.56	12.32±3.23	7.12±3.25
Hexadecane	1600	15.07±0.84[a]	6.36±6.30[a]	5.87±2.82[a]	0.99±1.26	16.68±3.61[a]	14.9±8.20[a]	0.20±0.45	0.50±1.33
Heptadecane[a]	1700	13.88±1.11	15.81±21.7	11.21±5.71	10.75±6.61	11.97±4.21	12.29±8.11	5.23±2.31	9.09±13.07
Octadecane	1800	34.29±2.30	-	-	1.90±3.14	38.52±9.01[a]	34.94±18.47	-	-
Nonadecane	1900	2.06±0.43	0.54±0.95	0.81±0.94	0.60±0.80	2.09±0.66	1.06±0.90	0.06±0.16	0.27±0.50
1-eicosene	1994	3.53±0.21	4.32±4.51[a]	4.28±3.43[a]	2.49±2.71	3.25±1.96[a]	4.07±2.22[a]	1.03±1.02	1.08±1.87
Eicosane	2000	1.71±0.21	1.68±2.02	1.86±2.49	0.71±0.89	1.01±0.78	1.46±0.79	0.26±0.36	0.37±0.71
11-Methyl heneicosane	2136	1.39±0.21	0.85±0.78	1.67±0.53	0.90±0.77	1.39±0.21	1.2±0.23	1.12±0.44	0.91±0.3
1-Docosene	2195	1.32±0.19	2.15±2.08	2.43±1.99	2.15±2.64	1.04±0.57	1.27±0.69	0.76±0.47	0.54±0.28
11-Methyltricosane	2333	0.81±0.14	0.49±0.65	1.24±0.36	0.69±0.59	0.81±0.13	0.55±0.20	0.86±0.19	0.71±0.27
Tetracosane	2400	-	-	0.24±0.40	-	-	-	1.04±0.28	6.22±2.48
Pentacosane	2500	-	-	-	-	-	-	1.98±0.45	0.96±0.40
Hexacosane	2600	-	-	-	-	-	-	2.33±0.55	1.03±0.41
Heptacosane	2700	1.94±0.17	5.46±4.19[a]	7.85±3.18[a]	13.11±5.78[a]	1.78±0.84	1.76±0.66	16.69±3.38[a]	6.39±2.60[a]
Octacosane	2800	-	-	-	-	-	-	1.45±0.60	0.51±0.26
4-Methyloctacosane	2849	-	-	-	-	-	-	-	0.12±0.38
Nonacosane	2900	-	-	0.28±0.63	2.46±1.41	-	0.4±0.46	3.42±0.85[a]	1.26±0.72
Hentriacontene	3094	2.20±0.92	7.86±5.23[a]	6.53±1.86[a]	6.72±2.56[a]	2.19±0.91	2.21±1.30	10.22±1.64[a]	9.86±2.57[a]
12.16-; 13.17-; 14.18-Dimethyldotriacontane[a]	3248	7.61±0.63	24.11±15.31	22.44±5.86	28.47±9.40	7.3±2.74	7.37±2.35	32.29±3.15	32.44±10.25
x.y –Dimethyldotriacontane	3259	-	6.10±4.88[a]	4.39±2.06[a]	5.42±3.22[a]	0.11±0.37	2.06±1.30	7.59±2.06[a]	10.98±2.90[a]
NI	1906	-	1.83±1.09	0.81±0.98	1.35±0.70	-	0.85±0.89	1.07±0.24	0.71±0.30
Percentage of identified compounds:		***100***	***98.17***	***99.18***	***98.64***	***100***	***99.14***	***98.93***	***99.28***

NI: Not identified.

n=10.

Table 3. Relative percentage area of compounds of *Odontomachus bauri* cuticle for samples exposed to different treatments and control.

		Control	**10ºC**	**15ºC**	**20ºC**	**25ºC**	**30ºC**	**35ºC**	**40ºC**
Compound	**ECL**	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD

Pentadecane[a]	1500	13.41±1.26	29.56±13.04	7.61±1.40	10.76±2.08	11.9±3.46	47.15±32.24	10.96±6.99	12.80±4.04
Hexadecane[a]	1600	12.42 ±0.68	29.83±6.14	10.97±2.04	6.76±3.62	12.71±1.83	9.14±13.4	10.67±3.89	10.21±2.94
Heptadecane	1700	9.73±0.67[a]	2.30±2.49	9.12±1.17[a]	6.66±1.94[a]	9.34±1.27[a]	2.35±4.07	5.80±3.59[a]	8.21±1.06[a]
Octadecane[a]	1800	41.02±2.40	14.97±13.53	36.3±2.58	29.58±3.29	41.95±2.24	10.32±17.35	36.46±7.06	24.15±2.92
9-nonadecene	1874	11.01±1.52	16.24±10.60[a]	4.54±3.10[a]	2.43±4.21	12.89±5.40[a]	12.02±17.09[a]	3.23±2.16[a]	2.86±2.43
Nonadecane	1900	3.02±0.08[a]	0.50±0.60	2.33±0.25	1.96±0.21	2.56±0.40	16.03±30.42[a]	2.57±0.39	2.39±1.25
1-eicosene	1994	3.93±0.36[a]	1.07±1.20	4.00±0.29[a]	3.70±0.51[a]	4.42±0.64[a]	1.27±2.24	5.63±0.79[a]	3.14±0.66[a]
Eicosane	2000	1.33±0.17	0.61±0.45	1.12±0.04	1.39±0.28	1.39±0.13	0.42±0.78	1.9±0.76	1.08±0.18
1-Docosene	2195	0.96±0.06	-	0.67±0.12	0.41±0.09	0.61±0.28	0.07±0.17	0.83±0.37	0.22±0.29
11-tricosene	2195	-	-	0.46±0.22	0.22±0.12	-	-	-	0.58±0.31
Tricosane	2300		0.10±0.21	0.10±0.09	0.15±0.33	-	-	-	0.04±0.12
2-methyldocosane	2328	0.55±0.13	0.93±0.31	0.52±0.04	3.16±5.33[a]	0.41±0.16	0.55±0.80	0.53±0.17	0.45±0.29
Tetracosane	2400	-	0.01±0.03	7.03±0.64[a]	9.73±6.19[a]	-	-	1.18±0.66	14.16±5.80[a]
Pentacosane	2500	-	2.99±5.24	0.02±0.03	0.04±0.12	-	-	-	-
Heptacosane	2700	-	-	0.47±0.28	1.18±1.25	-	-	0.82±0.56	-
Octacosane	2800	-	-	0.02±0.03	0.08±0.10	-	-	-	-
Nonacosane	2900	1.57±0.28	-	4.48±2.67[a]	4.98±5.01[a]	0.78±0.44	-	6.91±3.87[a]	2.64±1.50
5.11-dimethylnonacosane	2979	-	-	2.69±1.49	6.62±8.29[a]	-	-	6.6±4.93[a]	3.78±3.79[a]
Hentriacontane	3100	-	-	0.50±0.36	0.47±0.72	-	-	0.64±0.51	0.83±0.87
5-methylhentriacontane	3152	-	-	0.56±0.41	0.88±0.96	-	-	2.42±2.27	-
NI	1924	-	-	4.98±0.56[a]	6.78±1.38[a]	-	-	1.76±0.46	10.13±2.26[a]
NI	2112	1.02±0.06	0.87±0.52	0.88±0.10	1.13±0.22	0.98±0.04	0.64±0.78	0.94±0.18	0.93±0.23
NI	2125	-	-	0.66±0.11	0.87±0.18	-	-	0.11±0.13	1.24±0.26
Percentage of compounds identified:		***98.98***	***99.13***	***93.48***	***91.21***	***99.02***	***99.35***	***97.18***	***87.66***

#Control not subjected to temperature change. ECL = Equivalent Chain Length.

[a] major compounds for different temperatures.

NI: Not identified

n=10

Table 4. Relative percentage area of fatty acids presents in the cuticle of *Atta sexdens* submitted to different temperatures.

Compound	Control	10ºC	15ºC	20ºC	25ºC	30ºC	35ºC	40ºC
	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD
Lauric acid	0.52±0.01	0.52±0.01	0.51±0.01	0.52±0.01	0.52±0.01	0.52±0.01	0.52±0.01	0.52±0.01
Myristic acid	2.34±0.01	2.34±0.01	2.34±0.01	2.34±0.02	2.35±0.01	2.34±0.01	2.35±0.01	2.35±0.02
Palmitic acid [b]	34.64±0.04	34.61±0.08	34.63±0.05	34.69±0.07	34.60±0.08	34.61±0.08	34.65±0.03	34.62±0.05
Palmitoleic acid	3.59±0.02	3.57±0.01	3.60±0.01	3.59±0.02	3.59±0.06	3.58±0.02	3.6±0.01	3.58±0.01
Stearic acid	4.21±0.01	4.21±0.02	4.21±0.01	4.2±0.03	4.21±0.04	4.2±0.01	4.2±0.01	4.18±0.01
Oleic acid [b]	53.95±0.01	53.96±0.06	53.90±0.02	53.95±0.16	53.92±0.12	53.95±0.12	53.98±0.07	53.96±0.15

n=3; Control not subject to temperature variation.

[b]Major compounds for different temperatures>10%.

Table 5. Relative percentage area of fatty acids present in the cuticle of *Odontomachus bauri* submitted to different temperatures.

Compound	Control	10ºC	15ºC	20ºC	25ºC	30ºC	35ºC	40ºC
	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD	M±SD
Lauric acid	0.51±0.02	0.50±0.02	0.50±0.01	0.51±0.01	0.50±0.01	0.51±0.01	0.50±0.01	0.50±0.01
Myristic acid	2.40±0.05	2.42±0.02	2.34±0.01	2.42±0.04	2.43±0.01	2.43±0.02	2.43±0.02	2.42±0.03
Palmitic acid [b]	33.85±0.08	33.76±0.12	33.90±0.11	33.82±0.06	33.84±0.13	33.85±0.14	33.79±0.06	33.83±0.17
Palmitoleic acid	3.74±0.01	3.74±0.04	3.73±0.03	3.75±0.01	3.73±0.04	3.75±0.03	3.74±0.01	3.73±0.04
Stearic acid	4.02±0.03	4.00±0.02	4.00±0.02	3.99±0.02	4.03±0.04	4.01±0.06	4.01±0.02	3.98±0.05
Oleic acid [b]	54.6±0.03	54.62±0.06	54.61±0.08	54.57±0.14	54.56±0.09	54.53±0.15	54.6±0.12	54.55±0.15

n=3; Control not subject to temperature variation.

[b]Major compounds for different temperatures>10%.

Table 6. Relative percentage area of fatty acids present in the cuticle of *Ectatomma brunneum* submitted to different temperatures.

Compound	Control	10ºC	15ºC	20ºC	25ºC	30ºC	35ºC	40ºC
	M±SD	M± SD	M± SD	M± SD	M± SD	M± SD	M ± SD	M± SD
Lauric acid	0.50±0.02	0.50±0.01	0.49±0.01	0.49±0.01	0.49±0.01	0.50±0.01	0.49±0.01	0.51±0.01
Myristic acid	2.38±0.01	2.38±0.01	2.37±0.01	2.38±0.02	2.40±0.02	2.39±0.01	2.39±0.02	2.39±0.01
Palmitic acid [b]	34.68±0.03	34.65±0.01	34.67±0.02	34.66±0.04	34.68±0.02	34.67±0.02	34.66±0.05	34.70±0.02
Palmitoleic acid	3.59±0.01	3.58±0.01	3.60±0.01	3.59±0.02	3.59±0.01	3.60±0.01	3.60±0.01	3.62±0.02
Stearic acid	4.27±0.01	4.26±0.03	4.27±0.03	4.28±0.01	4.27±0.02	4.29±0.01	4.25±0.04	4.25±0.03
Oleic acid [b]	54.34±0.10	54.58±0.56	54.30±0.32	54.32±0.31	54.32±0.21	54.22±0.12	54.18±0.02	54.24±0.05

n=3; Control not subject to temperature variation.

[b]Major compounds for different temperatures>10%.

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Supplementary Material

Table 1. P values of the PERMANOVA analysis of the hydrocarbons in the samples under the effect of the different treatments pairwise compared in *Atta sexdens*.

	Control	10ºC	15ºC	20ºC	25ºC	30ºC	35ºC	40ºC
Control								
10ºC	0.0056							
15ºC	0.0028	0.0196						
20ºC	0.0028	0.0056	0.0028					
25ºC	0.0168	0.0028	0.0028	0.0028				
30ºC	0.0028	0.0028	0.0028	0.0028	0.0028			
35ºC	0.0028	0.0028	0.0028	0.0028	0.0028	0.0028		
40ºC	0.0028	0.0028	0.0028	1.0000	0.0028	0.0028	0.0028	

Table 2. P values of the PERMANOVA analysis of the hydrocarbons in the samples under the effect of the different treatments pairwise compared in *Odontomachus bauri*.

	Control	10ºC	15ºC	20ºC	25ºC	30ºC	35ºC	40ºC
Control								
10ºC	0.0056							
15ºC	0.0056	1.0000						
20ºC	0.0028	1.0000	1.0000					
25ºC	1.0000	0.0028	0.0084	0.0028				
30ºC	1.0000	0.0700	0.1344	0.0028	1.0000			
35ºC	0.0028	0.0112	0.0028	0.0084	0.0028	0.0028		
40ºC	0.0028	1.0000	0.0196	0.0056	0.0056	0.0056	0.0028	

Table 3. P values of the PERMANOVA analysis of the hydrocarbons in the samples under the effect of the different treatments pairwise compared in *Ectatomma brunneum*.

	Control	10ºC	15ºC	20ºC	25ºC	30ºC	35ºC	40ºC
Control								
10ºC	0.0056							
15ºC	0.0028	0.0028						
20ºC	0.0028	0.0056	0.1148					
25ºC	1.0000	0.0028	0.0028	0.0028				
30ºC	0.0028	0.4872	0.0028	0.0028	0.0028			
35ºC	0.0028	0.0056	0.0504	0.0336	0.0028	0.0084		
40ºC	0.0028	0.0056	0.0028	0.3752	0.0028	0.0084	0.0028	

CAPITULO 3 - Anti-inflammatory activity of the *Ectatomma brunneum* venom in rodents

1. Introduction

The Formicidae family, as well as the other members of the Hymenoptera order, developed their poison apparatus from the ovipositor of their aculeate ancestors [1]. In general, this apparatus consists in a simple or multiple glandular structure (venom gland), a reservoir where the venom is stored, and the convolute gland (internal structure of the reservoir), in addition to the stinger, which is responsible for the injection of venom [2]. The venom is an important tool for the ants due to their use of defense and / or predation, and can be also associated with others functions such as communication, defense against pathogens (colonial immunity) and as an herbicidal agent, characteristics that may explain the great evolutionary success of this group [3].

The venoms of ant species are mainly composed of a complex mixture of proteins, a group of polycationic peptides and toxins of low molecular mass [4]. In contrast to other venomous animals, the poison of the Formicidae family are still a little studied, a fact that can be attributed to the low amount of this glandular material in the reservoir of these insects [4;3]. However, despite difficulties in obtaining sufficient material for studies of greater complexity, the venom of different ant species have been presented several bioactive elements that can be considered a potential pharmacological and therapeutic agent [5].

The advances within the study of proteins in recent decades have brought a new vision to the bioprospecting area, allowing new drug opportunities [4]. In this context, ant venom, as well as other Hymenoptera, have been intensively studied and submitted to modern studies of their profiles, making possible the development of new potency bioactive models for new drugs [4; 3].

Several venoms derived from animals and insect presents anti-inflammatory action [6;7;8]. The most studied arthropods venom as anti-inflammatory natural venom is bee venom. The main component responsible for bee venom anti-inflammatory action is melittin. Melittin is the major component of bee venom has pharmacological effects on various diseases and biological processes [9]. Because this action of bee venom and melittin, this study was encouraged thought testing ant venoms against inflammatory process.

Inflammatory process is complex and involves vascular, cellular components and a variety of soluble substances that presents characteristic clinical signs: flushing, heat, edema, pain and loss of function [10]. The purpose of this process is eliminate aggressive agents, prevent and cure tissue injury [11;10]. It is well established that polymorphonuclear leukocyte (PMN) recruitment is essential for inflammation development acting as the first line of defense [12],

and involves the participation of several mediators, such as prostanoids, cytokines and other substances produced [13].

Biological activities in ants' venoms have already been described previously. An assay using the *Diponera Quadriceps* specie showed that venom has presented antinociceptive, antimicrobial, neuroprotective and anti-inflammatory activity [14]. Anti-inflammatory activity in ant venom has also been reported for *Pseudomyrmex triplarinus*, and this activity is attributed to a series of six proteins called myrmexin I-IV and suggesting that these can be considered a new class of anti-inflammatory proteins [15].

Ectatomma brunneum is an ant that belongs to the genus *Ectatomma* and has preference for areas of open vegetation, but is found in cultivated vegetation of some South American countries, such as Panama, Argentina and Brazil [16]. The ecological and taxonomic diversity of the ant venoms suggests that these insects can be a source of bioactive molecules that could be used in the search of new drugs [3]. This study aims to identify the proteins present and demonstrate anti-inflammatory activity in rodents of the venom of *E. brunneum.*

2. Material and methods

2.1. Collection and obtaining of the E. brunneum venom

Workers of *E. brunneum* were collected in the municipality of Dourados (State of Mato Grosso do Sul - MS, Brazil - 22 ° 13'24.39 "S, 54 ° 54'44.53" W), in foraging activity. The whole procedure was performed by random capture in order to increase the representativeness of the forages and reduce the variation of the composition of the venom as a function of the variation by polytheism [3] and age, since this colonial function is performed by older individuals [17].

In the laboratory, the ants were anesthetized by freezing and dissected using a stereomicroscope (stereomicroscope S6D, Leica, Germany). During the dissection, the venom reservoirs were placed in eppendorfs containing 50 μL of ultrapure water, remaining refrigerated throughout the process. After the extraction step, the material was centrifuged (NT 805, Novatecnica, Brazil) at 13700 x g for 10 minutes at 4ºC, to separate the reservoir membrane from the venom. The supernatant was then separated and frozen at -20 ° C for further analysis. For the study of the biological activity, the crude *Ectatomma brunneum* venom (EBV) was lyophilized to prepare the tested concentrations.

2.2. Proteomic analysis

The concentration of total proteins present in the PEB was determined by the method of Bradford [19] using bovine serum albumin as standard. The venom samples (100 μg protein) were applied by rehydration in 7 cm immobilized pH gradient (IPG, GE Healthcare - USA) tapes and pH 3-10 linear for 10 hours in solution of rehydration containing 2% (v / v) IPG buffer pH 3-10, 40 mM Dithiothreitol (DTT) and DeStreak solution (GE Healthcare - USA). The first dimension of the gel was performed on IPGphor Ettan III (GE Healthcare - USA) equipment with linear mode voltage (300 V for 12 hours, 1000 V for 30 minutes, 5000 V for 2 hours, 5000 V for 1 hour and 200 V for 1 hour). After the isoelectric concentration, the IPG ribbons were incubated in equilibrium solution containing 6 M urea, 75 mM Hydroxymethyl Aminomethane Hydrochloride (Tris-HCl) (pH 8.8), 29% (v / v) glycerol, 2% (w / v) Dodecyl Sulfate (SDS) and 0.002% (v / v) bromophenol blue. The tapes were then maintained for 15 minutes under stirring in 1% (w / v) DTT and in 2.5% (w / v) iodacetamide for a further 15 minutes.

The second dimension (2D) was performed from 14% polyacrylamide gel, SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) according to the methodology described by Laemmli [20] and methodological instructions of the Mini-Protean Tetra Cell USA), using the Mini Protean II (Bio-Rad - USA) vertical vat, under 80 W electrical current for approximately 100 minutes. Subsequently the gels were stained for 24 hours with Coomassie Brilliant Blue G-250, as described by Santos et al. [21] and stored at 21 ° C in preservation solution, 5% (v / v) acetic acid. Scanning of the 2D gel was performed on Image Scanner III (GE-Healthcare, USA) in 16-bit transparency mode in red-blue colors and 600 dpi (dots per inch) resolution for documentation. The images were analyzed using Image Master 2D Platinum v.7 software (GE Healthcare, USA).

The spots obtained from the two-dimensional gel were excised and dehydrated in a solution containing 50% (v / v) acetonitrile (ACN) and 20 mM ammonium bicarbonate (AMBIC) (pH 8.0). After dehydration, the gels were sequentially incubated in 65 mM DTT solution for 30 minutes at 56 ºC and in 200 mM iodoacetamide solution for 30 minutes (in the dark) at room temperature, followed by washing in 100 mM AMBIC (pH 8.0), and 100% (v / v) ACN. The fragments of the gels were treated with 10% (v / v) ACN solution and 40 mM AMBIC containing trypsin (Sequencing Grade Modified Assayed Trypsin, Promega, USA) at a concentration of 25 ng / μL and incubated at 37 °C for 16 hours. Extraction of the purified compounds into the fragments of the gels was done by the addition of 5% (v / v) formic acid

and 50% (v / v) ACN. The extract obtained was concentrated in vacuo and solubilized in 10 µl of 0.1% (v / v) trifluoroacetic acid (TFA). The samples were desalted using ZipTip tips with C18 resin (Pipette Tips for Sample Preparation- Millipore, USA), according to the manufacturer's instructions, vacuum dried and prepared for MALDI-TOF / TOF mass spectrometry (matrix-assisted laser desorption / ionization - time-of-flight / time-of-flight).

The material obtained from the gel digestion was solubilized in 10 µl of 0.1% (v / v) TFA solution and had its matrix mixed (2.5 mg / ml α-cyano-4-hydroxy cinnamic matrix). The matrix was prepared in 50% (v / v) ACN and 0.1% (v / v) TFA in the ratio of 1: 1 (sample: matrix). Aliquots were applied on MTP Anchor ChipTM 600/384 TF steel plate (Bruker Daltonics, USA) and subjected to MALDI-TOF / TOF type mass spectrometry on Ultraflex III apparatus (Bruker Daltonics, USA). For the first peptidic fragmentation (MS / MS), the spectra were acquired in reflector mode (LPPepMix), with detection range of 500-5000 Da. The method calibration standard was used Peptide Calibration Standard II (Bruker Daltonics, Germany). For the second fragmentation (MS/MS) the spectra were obtained in LIFT mode in the mass range at v40-4000 Da. Spectrum acquisition mode was reflective, ion polarity was positive, voltage at the source of ionization was 25 kV, number of shots per shot was 400 and verage laser intensity was 30%.

The mass spectra obtained from MALDI-TOF / TOF were processed using FlexAnalysis 3.3 software (Bruker Daltonics, USA) and submitted to two research software: MASCOT (Matrix Science Ltd, UK; Peptide Mass Fingerprint-PMF and MS / MS Ion Search) and Peaks DB 7.0 (Bioinformatic Solutions Inc., Canada). The identifications obtained from the MASCOT software were validated by Scaffold 4.0 software (Proteome Software Inc., USA). The identifications from Peaks 7.0 were also validated assuming the FDR (False Discovery Rate) equal to 0.0%. The protein and peptide sequences deposited in NCBInr and Swissprot were used, assuming an error of 0.5 Da. Reactions of cysteine carbamidomethylation and oxidation of methionine were used as fixed and variable modifications, respectively. Metazoa (Animals), Insecta, Formicidae, and "proteins from animal venom" (mollusks, snakes, insects, arachnids, amphibians) were selected as taxon for entry into the databases.

Sequences suggested by the PEAKS Studio 7.0 program (Bioinformatics Solutions Inc., Canada) that presented Average Local Confidence (ALC) of 70% or higher were submitted to the MS Blast research database [http: //genetics.bwh. harvard.edu/msblast/]. Sequences that

showed significant alignments with proteins already described in ants and animal venom were considered as positive identification.

2.3. Study of the anti-inflammatory activity of the Ectatomma brunneum venom (EBV)

2.3.1. Animals

Female Swiss mice (weighing between 20-30g) from the Central Biotherm of the Federal University of Grande Dourados were used. The animals were kept under standard animal husbandry conditions, with a temperature of 22 ± 2 ºC and a light / dark cycle of 12 hours, with *ad libitum* water and food. The experimental protocol was submitted and approved by the Ethics Committee of Experimental Animals of the Universidade Federal da Grande Dourados (CEUA/UFGD 03-2018).

2.3.2. Carrageenan-induced pleurisy in mice model

To detect the dose that EBV (3, 30, or 300 μg/kg, n = 6) reduced by intraperitoneal route the leukocyte migration the model of pleurisy was chosen. Different groups of animals were treated intraperitoneally with EBV (3, 30 and 300 μg/kg), dexamethasone (used as a reference drug at a dose of 1 mg / kg, subcutaneously), or vehicle. One hour after the treatment, the animals received intrapleural injection of carrageenan, 300 μg diluted in 0,1 mL of sterile saline, according to the technique described by Vinegar et al. [22], the animals of the naive group were injected with intrapleural saline solution only. After 4 hours of carrageenan injection, the animals were anesthetized and submitted to euthanasia. The pleural cavity was washed with 0.5 mL of PBS buffer, the material was centrifuged for 10 minutes at 2500 rpm and the pellet resuspended in 0.5 mL of PBS. An aliquot of this cell suspension was used for the determination of total leukocytes in the Neubauer chamber, using Turk's liquid. The results were expressed as number of leukocytes per mL of pleural exudate.

2.3.3. Zymosan-induced arthritis model in mice

The zymosan-induced arthritis model was performed as previously described in the literature [23]. To accomplish this model, a solution containing 200 μg of zymosan diluted in sterile saline was prepared for application in the joint cavity of the mice. The right knee joints of the mice received an intra-articular (i.a.) injection of the zymosan solution, and the left

knee joints of the animals were injected with the same volume of sterile saline. One hour prior to the i.a. injection of zymosan or saline, the animals were treated intraperitoneally with vehicle, EBV (3 μg / kg) or dexamethasone (used as a reference drug at the dose of 1 mg / kg subcutaneously). Hypernociception (3 and 4 hours after arthritis induction) and articular edema formation (4 and 6 hours after arthritis induction) were determined. Six hours after the induction of arthritis, the animals were anesthetized and euthanized to perform the total leukocyte count in the joint cavity of the animals.

2.3.4. Mechanical hypernociception

Mechanical stimulation was exerted on the plantar region of the right hind paws of the animals (who received i.a. injection of zymosan). The electronic device (Von Frey) records the sufficient pressure in grams to cause a reaction described as a flexion of the paw followed by a flinch after withdrawal of the paw in contact with the apparatus. For this, the animals were individually placed in transparent acrylic compartments (9 x 7 x 11 cm), located on a raised wire platform to allow access to the ventral surface of the hind paws. Mechanical hypernociception (degree of sensitivity to mechanical stimuli) was evaluated into 3 and 4 hours after arthritis induction.

2.3.5. Analysis of the joint edema formation

Knee joint edema was assessed by measuring the right knee diameter difference (i.a. injection of zymosan) and left knee, a digital micrometer was used to measure edema. Measurement of edema was performed at the 4 and 6 hour time points after arthritis induction. For the measurement of the joints the animal was carefully immobilized and the measurement of the knee diameter was performed. The formation of edema was expressed in micrometers, being the difference between the measurement of the right and left knees of each animal.

2.3.6. Determination of migration of leukocytes to the joint cavity

For the counting of leukocytes in the joint cavity, the knee joint was exposed by surgical incision and washed 2 times with 5 μL of PBS / EDTA (pH 7.4). The obtained joint wash was diluted in PBS / EDTA to a final volume of 50 μL, for subsequent realization of total and differential leukocyte counts. Total leukocyte count was performed in Neubauer

chamber by dilution of the sample in Turk's liquid. The results were expressed as number of leukocytes per well.

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software. Data were expressed as mean ± standard error of the mean (SEM) for each group. The results were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. E. brunneum venom proteomic

The fractionation by 2D electrophoresis presented approximately 100 spots with isoelectric points ranging from 4.70 to 9.43, and molecular masses ranging from 3 to 299 KDa. As of these, four expressive spots were excised and analyzed by MALDI-TOF.

The proteins of the EBV were identified based on the database UniProt database (2017). The four proteins identified were the multifunctional dipeptidylpeptidase 4 (DPP-4) (Access Code E2AF09_CAMFO); Tensin (Access code G7Y462_CLOSI); D-glucuronyl C5-epimerase (Access code A0A026WTR0_CERBI) and Focal adhesion kinase 1 (Accessorcode: T2MDJ8). The dipeptidyl peptidase 4 multifunctional protein was found in the Camponotus floridanus ant, the Tensin protein was identified in other venomous animals, the D-glucuronyl C5-epimerase protein was identified in the Cerapachys biroi ant; while the protein Focal adhesion kinase 1 was identified in a freshwater polyp Hydra vulgaris (UniProt database, 2017).

3.2. Treatment with EBV reduces leukocyte recruitment in the carrageenan-induced pleurisy model

After 4 hours of carrageenan induction of pleurisy, we observed an evident inflammatory response, characterized by recruitment and consequent increase in the number of leukocytes ($7.51 \pm 1.65 \times 10^6$ cells / well) compared to group receiving saline injection alone ($1.22 \pm 0.91 \times 10^6$ cells / well). Pretreatment with EBV at doses of 3, 30 and 300 μg / kg significantly reduced leukocyte recruitment by 49.40%, 26.76% and 51.79%, respectively,

compared to the control group (Figure 1). Dexamethasone, which was used as a reference drug, reduced leukocyte recruitment by 82.8%, compared to the control group.

3.3. Treatment with EBV reduces nociception in zymosan-induced arthritis model

Figure 2 shows that the zymosan injection promoted an increase in mechanical sensitivity, compared with the naive group, at the time of 3 hours (tolerance of 0.61 ± 0.31 g for the control group and 5, 75 ± 1.38 g for the naive group) (Figure 2A) and 4 hours (tolerance of 0.56 ± 0.19 g for the control group and 5.8 ± 1.24 g for the naive group) (Figure 2B) after the induction of arthritis. Treatment with EBV was able to decrease the sensitivity threshold only 4 hours after the induction of arthritis (Figure 2B), since dexamethasone, which was used as the reference drug, decreased the nociception threshold at times of 3 and 4 hours after arthritis induction (Figure 2A and 2B).

3.4. Treatment with EBV reduces edema formation in zymosan-induced arthritis model

Figure 3 shows the influence of treatment with EBV on the formation of edema in 4 (Figure 3A) and 6 (Figure 3B) hours after the induction of arthritis. Treatment with EBV reduced the formation of edema formation by 31.45% 4 hours after the induction of arthritis, compared to the control group. Dexamethasone was used as reference drug, reduced edema formation by 58.87% at the same time (Figure 3A). At 6 hours after arthritis induction, treatment with EBV was able to reduce edema formation by 66.14% when compared to the control group, and dexamethasone reduced edema formation by 51.61% (Figure 3B).

3.5. EBV reduces cellular influx into the joint cavity of the mice after zymosan-induced arthritis

The cellular infiltrate in the joint cavity of the mice was evaluated 6 hours after the induction of arthritis. Six hours after injection of zymosan (200 μg / well) a significant increase in the number of leukocytes in the joint cavity of the control group (480 ± 33.17 x 10^3) was observed in relation to the group that received only saline injection (127.5 ± 10.61 x 10^3). As seen in Figure 4, treatment with EBV reduced the influx of leukocytes into the joint cavity by 47.65% compared to vehicle treated animals, a similar response was observed in dexamethasone treatment, which reduced the influx of leukocytes at 55.46%.

4. Discussion

The inflammatory response is characterized by some classic signs, such as: pain, heat, flushing and edema, being an important defense mechanism against invading pathogens [10]. In addition, immune cells play a key role in the inflammatory process, forming the first line of defense against microbial invasions [24]. It is well established that cyclooxygenase-derived mediators (COX), such as prostaglandins, are essential in the initiation and maintenance of the inflammatory response [25]. The anti-inflammatory drugs COX inhibitors are effective anti-inflammatory drugs, since they block the synthesis of prostaglandins; however these drugs promote a series of adverse events in users, such as cardiovascular complications and gastric lesions [26]. Thus, the search for other alternative substances with anti-inflammatory activity has been increasing in recent years.

Besides the ants use the venom for defense and protection, the use of this compounds as a source of potential bioactive toxins are still unexplored, what results in the improvement of the new techniques of analyses and isolation of the peptides contained in the venom of these insects, making possible the development of new drugs and bioinsecticides [27]. In an unprecedented way, this study demonstrated anti-inflammatory properties of EBV, and its results showed that this venom reduces the formation of edema, inflammatory pain and the recruitment of leukocytes in the inflammatory process.

In the carrageenan-induced pleurisy model in mice, treatment with EBV promoted a decrease in leukocyte recruitment compared to the control group at all doses (3, 30 and 300 μg / kg), similar to that observed with dexamethasone (drug anti-inflammatory drug) at a dose of 1 mg / kg. The inflammatory response induced by carrageenan presented peaks within 4 hours after induction, that it is characterized by cellular activation and the release of various inflammatory mediators, such as proinflammatory cytokines (such as tumor necrosis factor (TNF), interleukin-1 (IL-1) and interleukin-6 (IL-6)), nitric oxide and prostanoids originating from COX [28]. These released mediators induce the expression of vascular adhesion molecules that participate in the process of migration of leukocytes from the intravascular environment to the tissue injured in inflammation. Observing our results, we may suggest that EBV may be reducing some of the inflammatory mediators released by the inflammatory response induced by carrageenan.

As in the carrageenan-induced pleurisy model, all doses of EBV tested showed satisfactory activity, and the dose of 3 μg / kg was chosen for the zymosan-induced arthritis model, since it was the lowest dose that was effective in our experiment.

In addition to leukocyte recruitment, pain and edema are characteristic components of the inflammatory response [29]. Thus, in the zymosan-induced arthritis model, we evaluated hyperalgesia, edema formation and leukocyte recruitment in the joint cavity of the animals' knees. Zymosan is a polysaccharide extracted from the cell wall of the fungus *Saccharomyces cerevisiae*. This polysaccharide is a TLR-1, TLR-2 and TLR-6 cellular receptor agonist, present in immune cells, and promotes activation of the NF-kB transcription factor, which regulates the expression of various proinflammatory cytokines, including TNF, IL-1, IL-6, and nitric oxide [30;31].

This model has been extensively used in the study of the effect of natural and synthetic products on the inflammatory response, since the lesions promoted in this model resemble the joint lesions present in human rheumatoid arthritis [32]. Intra-articular injection of zymosan induces hypernociception with local edema between the 3 rd and 4 th hour after induction. Six hours after the induction of arthritis, a peak leukocyte infiltration occurs in the joint cavity [33;34].

Our results demonstrated that the treatment with EBV (3 μg / kg) presented an antihyperalgesic activity only 4 hours after the induction of joint inflammation, an effect also observed in the treatment with dexamethasone, however, in the time of 3 hours after the induction of the articulate inflammation this effect was not observed. Also in this model, treatment with EBV reduced the formation of edema at times 4 and 6 hours after induction of arthritis by zymosan, as well as observed in the treatment with dexamethasone. In addition, leukocyte recruitment was reduced after 6 hours of arthritis induction in animals treated with EBV, as observed in the pleurisy model.

In human rheumatoid arthritis, there is joint inflammation, leukocyte infiltration in the joint cavity, formation of joint edema, and lesions in the synovial articular membranes [32]. Therefore, PEB can be a promising compound to be studied and investigated as the possible treatment of rheumatoid arthritis and other types of inflammation.

The identified proteins are reported in the literature because of their activities presented such as muscle regeneration and regulation of anti-inflammatory function in bone marrow cells [35;36]. D-glucuronyl C5-epimerase, for example, can suppress the proliferation of breast and lung cancer cells [37;38].

Some proteins present in insect venoms have proinflammatory role described in the literature (such as DPP-4, present in PEB in our study) [39], and others, such as focal adhesion kinase suppressed production of inflammatory cytokines by activated immune cells, evidencing this as an anti-inflammatory potential [36]. While the Tensin has an important biological role as a kidney function aid and wound healing, for example [35]. However, as we are working with the crude venom, we can suggest that the constituents present may be acting synergistically, culminating with the anti-inflammatory activity found.

Dexamethasone, the reference drug used in research, has the effect of reducing inflammatory mediators, besides reducing the expression of vascular adhesion molecules [40], but the effective dose of EBV in the assay was much lower than the dose of dexamethasone, which leads us to believe that EBV may be a promising bioactive substance in the treatment of inflammatory conditions if studied in more depth.

Studies related to the characterization of anti-inflammatory substances in ant venoms are still very limited, and the mechanisms of anti-inflammatory activity act of different ways in each protein [15]. However, these studies point to ant venom as a promising source of bioactive compounds and further studies with the purpose of discovering the mechanisms of action of PEB should be considered in future studies

5. Conclusion

In conclusion, in vivo treatment with EBV showed anti-inflammatory activity, reducing leukocyte migration and edema formation, besides presenting an antihyperalgesic activity in the experimental model of arthritis. The mechanism of action may be related to reduced production of inflammatory mediators. More studies are needed to elucidate the mechanism of action of EBV.

Figures

Figure 1. Effect of treatment with EBV on the leukocytes recruitment in carrageenan-induced pleurisy in mice, 4 hours after induction. * $p < 0.05$ compared to the control group. #$p < 0.05$ compared to the naive group (ANOVA, Newman-Keuls test). EBV: *E. brunneum* venom. DEX: dexamethasone.

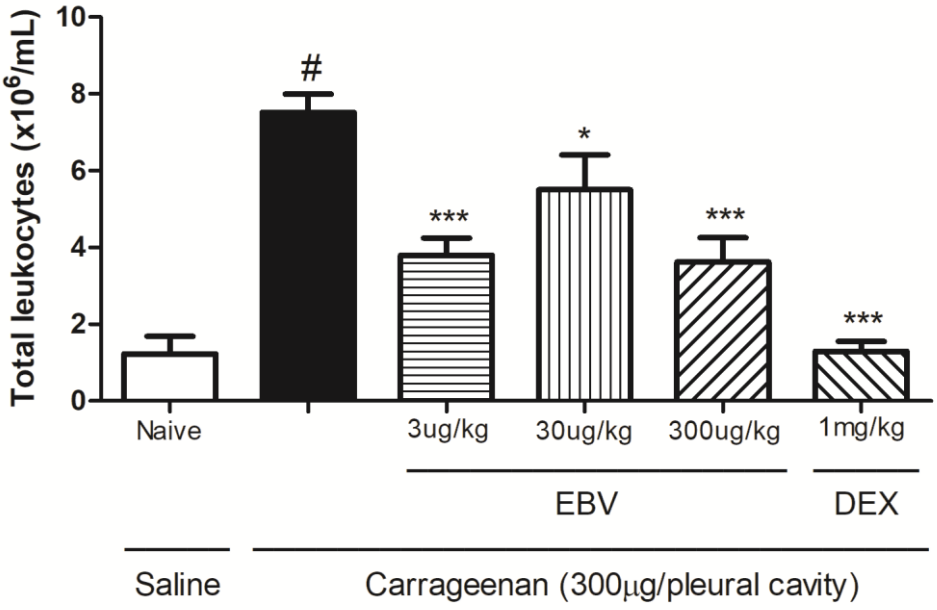


Figure 2. Effect of the treatment with EBV on the nociception in zymosan-induced arthritis model in mice, 3 (A) and 4 (B) hours after induction. * p <0.05 compared to the control group. #p <0.05 compared to the naive group (ANOVA, Newman-Keuls test). EBV: *E. brunneum* venom. DEX: dexamethasone.

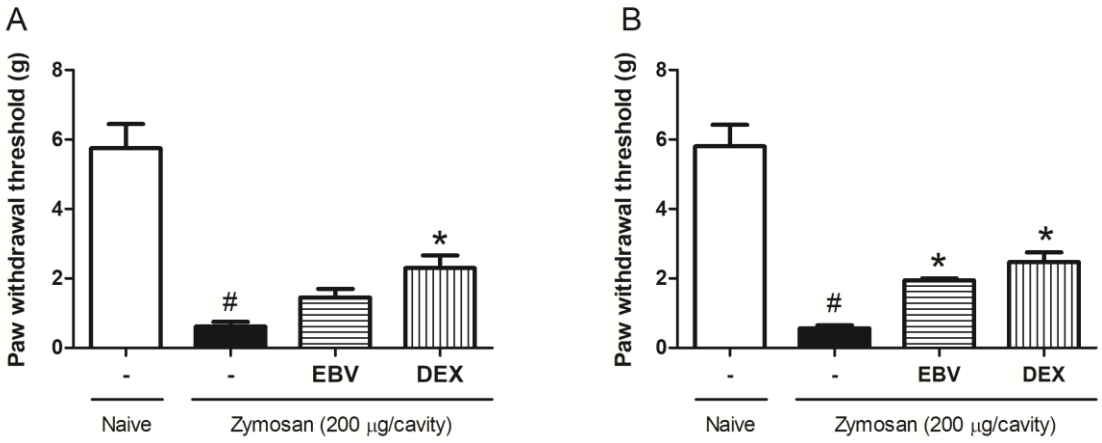


Figure 3. Effect of treatment with EBV on the formation of joint edema in zymosan-induced arthritis model in mice, 4 (A) and 6 (B) hours after induction. * p <0.05 compared to the control group. (ANOVA, Newman-Keuls test). EBV: *E. brunneum* venom. DEX: dexamethasone.

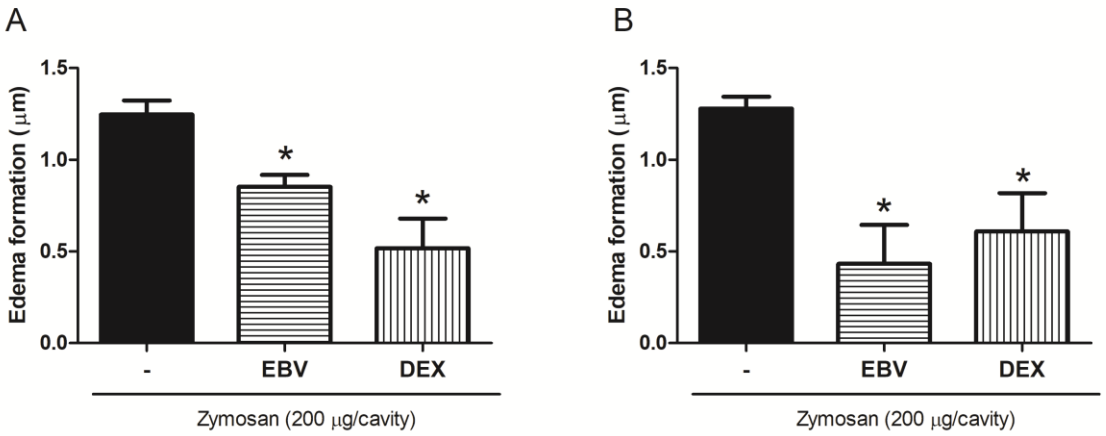
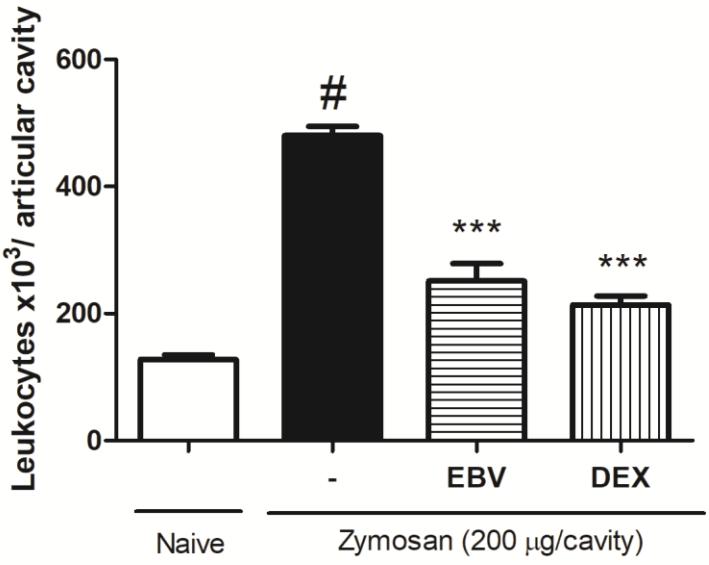


Figure 4. Effect of treatment with EBV on the number of leukocytes migrated to the joint cavity 6 hours after arthritis induction. Values represent mean ± SEM. * p <0.05 compared to the naive (saline) control group. #p <0.05 compared to the positive control group. (ANOVA, Tukey's test). EBV: *E.brunneum* venom. DEX: dexamethasone.



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