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**Custos da defesa química em opiliões  
(Arachnida: Opiliones)**

**Costs of chemical defense in harvestmen  
(Arachnida: Opiliones)**

Orientador: Dr. Glauco Machado

São Paulo  
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	Tese apresentada ao Instituto de Biociências da Universidade de São Paulo como parte dos requisitos para obtenção do título de Doutor em Ciências, na área "Ecologia".
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Orientador: Prof. Dr. Glauco Machado  
Departamento de Ecologia, Universidade de São Paulo

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by Bill Waterson

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## Resumo

As formas de defesa encontradas na natureza são incrivelmente diversas e envolvem estratégias que minimizam as chances de encontro com o predador ou as chances de escape diante de um ataque. A liberação de substâncias químicas é uma forma de defesa amplamente difundida entre artrópodes e vários estudos já demonstraram sua eficiência contra o ataque de predadores. Apesar de trazerem benefícios óbvios em termos de defesa, a produção de vários desses compostos químicos deve ser custosa para as presas. Esta tese teve como objetivo geral explorar os custos da produção de defesas químicas em um grupo particular de artrópodes, os opiliões. No capítulo 1, investigamos como a produção de ovos pode comprometer a produção de defesas químicas (benzoquinonas) e quais as conseqüências da redução do volume de secreção liberada sobre a capacidade de defesa das fêmeas de *Acutisoma longipes*. Nossos resultados apontam que a quantidade de secreção produzida por fêmeas ovígeras é quase 50% inferior à das fêmeas não-ovígeras. Como conseqüência, a secreção liberada por fêmeas ovígeras é menos eficiente em deter formigas e aranhas do que a secreção liberada por fêmeas não-ovígeras. No capítulo 2, investigamos como a quantidade e a qualidade da dieta influenciam a produção de defesas químicas (benzoquinonas) em *Magnispina neptunus*. Demonstramos que a produção de defesas químicas é condição dependente, pois indivíduos bem alimentados produziram mais secreção que indivíduos mal alimentados. Entretanto, indivíduos que receberam aporte extra dos precursores da secreção não incrementaram nem a quantidade total nem a concentração da secreção. Por fim, o capítulo 3 teve como objetivo investigar possíveis demandas conflitantes (*trade-offs*) entre o esforço de acasalamento (produção de armamentos) e o esforço somático (defesas químicas) de machos e fêmeas em um clado de opiliões neotropicais. Usando uma abordagem comparativa, mostramos que fêmeas produzem mais defesas químicas (benzoquinonas) que machos, mas não há uma relação negativa entre o dimorfismo sexual morfológico e o dimorfismo na quantidade de defesas químicas produzidas por machos e fêmeas. Coletivamente, os resultados obtidos aqui apontam que a produção de defesas químicas em opiliões é custosa e que está sujeita a demandas conflitantes com outros componentes de aptidão.

## Abstract

The forms of animal defenses found in nature are incredibly diverse and comprise many strategies that minimize the chances of a prey being detected or attacked by a predator. The emission of chemical secretions is a widespread defense among arthropods, and several studies have already demonstrated the efficiency of chemical defenses against predation. Although chemical defenses confer obvious survival benefits, the production of deterrent compounds may be costly for the individuals. The main goal of this thesis was to evaluate the costs of producing chemical defenses in a particular arthropod group, the harvestmen. In chapter 1 we investigated how egg production may compromise the production of chemical defenses (benzoquinones) by females of *Acutisoma longipes*, and whether a reduction in the amount of secretions released by ovigerous females make them more vulnerable to predation. Our results indicate that ovigerous females produce almost 50% less secretion than non-ovigerous females. Moreover, the low amount of secretion released by ovigerous females is less efficient than the amount released by non-ovigerous females in deterring ants and spiders. In chapter 2 we investigated how the quantity and quality of food influence the production of chemical defenses (benzoquinones) in *Magnispina neptunus*. We demonstrate that the production of chemical defenses is clearly condition dependent because well fed individuals produced more secretion than poorly fed individuals. However, individuals that received in the diet an input of the benzoquinones' precursor did not show an increase in the amount and concentration of secretion released. Finally, in chapter 3 we investigated possible trade-offs between mating effort (i.e., investment in weaponry) and somatic effort (i.e., investment in chemical secretions) in males and females of several harvestman species belonging to the family Gonyleptidae. Using a comparative approach, we showed that females consistently produce more secretion than males, but there is no negative relationship between morphological sexual dimorphism and sexual dimorphism in the amount of secretion released by males and females. Taken together, our findings indicate that the production of chemical defenses in harvestmen is costly and is under allocation trade-offs with other fitness components.

## Introdução geral

Todos os animais precisam se defender de predadores em pelo menos uma parte do seu ciclo de vida. As formas de defesa encontradas na natureza são incrivelmente diversas e incluem tanto adaptações morfológicas quanto comportamentais que aumentam a probabilidade de sobrevivência das presas. Em geral, as primeiras linhas defensivas simplesmente diminuem a chance de uma presa ser encontrada pelo predador e envolvem defesas como a camuflagem e a redução da locomoção ou da produção de ruídos (Edmunds, 1974; Ruxton *et al.*, 2004). Entretanto, mesmo presas que apresentam tais defesas podem se deparar com predadores. Nessa situação, outro conjunto de defesas é empregado, envolvendo mecanismos de desencorajamento do ataque pelo predador. Por exemplo, presas podem assustar o predador exibindo ocelos ou colorações conspícuas, podem ameaçar o predador parecendo maiores ou atacando-o com pinças, garras ou espinhos ou podem ainda se fingir de mortas e desencorajar o predador pelo risco de consumo de um alimento em decomposição (Edmunds, 1974; Ruxton *et al.*, 2004). Eventualmente, mesmo usando linhas secundárias de defesa, a presa pode ser capturada e é neste momento que uma terceira linha defensiva, normalmente com alto custo energético, é empregada: a liberação de substâncias químicas que podem ser tóxicas ou irritantes para o predador (Edmunds, 1974; Berenbaum, 1995).

Existe uma diversidade enorme de compostos químicos utilizados como defesa pelos animais. Entre os artrópodes, os compostos secretados incluem ácidos, aldeídos, cetonas, ésteres, hidrocarbonetos, lactonas, fenóis e benzoquinonas (Blum, 1981; Whitman *et al.*, 1990; Eisner *et al.*, 2005). Apesar de trazerem benefícios óbvios em termos de defesa, a produção de vários desses compostos químicos provavelmente é custosa para as presas (e.g., Cohen, 1985; Zalucki *et al.*, 2001; Skelhorn & Ruxton, 2007). Portanto, mesmo que defesas químicas sejam eficientes contra predadores, um alto custo energético em sua produção poderia explicar porque elas são comumente utilizadas como último recurso diante do ataque de um predador (Whitman *et al.*, 1990). Adicionalmente, adaptações na forma de liberação de defesas químicas permitem que elas sejam usadas com máxima eficácia e mínimo desperdício. Espécies que liberam secreção na forma de *spray*, por exemplo, geralmente possuem a capacidade de apontá-lo diretamente para o corpo do predador (e.g., Eisner & Meinwald, 1966; Gnaspini & Hara, 2007). Algumas espécies possuem mais de uma glândula de secreção defensiva espalhada pelo corpo e, dependendo do local de ataque do predador, a presa pode utilizar o conteúdo de uma ou mais glândulas de forma a maximizar a defesa e minimizar os possíveis custos da liberação maciça de secreção. Esse é o caso dos diplópodes,

que possuem um par de glândulas defensivas por segmento e, se o estímulo do predador for localizado, as glândulas mais próximas respondem liberando secreção (e.g., Eisner & Meinwald, 1966).

Uma forma de diminuir os possíveis custos associados às defesas químicas é o seqüestro de compostos a partir da dieta, o que pode ser feito consumindo tanto animais quanto plantas, que são os reais produtores dos metabólitos defensivos (Lindquist & Hay, 1995). Grande parte dos estudos que têm como objetivo relacionar a dieta com a eficácia da defesa foi feita com invertebrados que se alimentam de plantas das quais seqüestram seus compostos químicos (Jones *et al.*, 1987; Brower *et al.*, 1982, 1984). Um caso interessante é o da borboleta *Cosmosoma myrodora*, em que machos seqüestram substâncias químicas defensivas de plantas e, por meio do acasalamento, as fêmeas recebem estas substâncias dos machos e adquirem proteção química (Conner *et al.*, 2000). Invertebrados que seqüestram compostos químicos são relativamente bem estudados e os custos associados às defesas químicas são considerados baixos. Para invertebrados que produzem seus próprios compostos defensivos, entretanto, nosso conhecimento sobre os efeitos da dieta é mais limitado. Para as espécies autógenas, ou seja, aquelas que produzem seus próprios compostos defensivos, a classe de compostos produzidos deve determinar a composição ideal da dieta (e.g., Byers, 2015). Proteínas e carboidratos, por exemplo, fornecem aminoácidos e energia, que são limitantes tanto para o crescimento quanto para a montagem de defesas imunológicas e químicas (Schoonhoven *et al.*, 2005; Karasov & Martínez del Rio, 2007; Roeder & Behmer, 2014). Portanto, a qualidade do alimento que os indivíduos consomem deve estar diretamente relacionada com a qualidade e a quantidade de defesas que eles irão produzir.

A alocação de energia e recursos para uma atividade biológica comumente implica em redução na energia e recursos disponíveis para outras atividades, gerando o que se conhece como demandas conflitantes ou *trade-offs* (Harshman & Zera, 2007). O investimento em crescimento, por exemplo, pode depreciar o investimento em reprodução e defesa tanto em plantas quanto em animais (Roff, 2002). De fato, um estudo com sete espécies de esponjas do Caribe demonstrou haver uma relação negativa entre a taxa de crescimento e resistência à predação promovida por espículas e compostos químicos (Leong & Pawlik, 2010). Dessa forma, a maneira pela qual a alocação de energia e recursos é feita pode ter influência direta sobre a aptidão dos indivíduos (Magrath & Kondeur, 2003). Além disso, a seleção sexual impõe pressões seletivas diferentes sobre machos e fêmeas, resultando em uma clara dicotomia em relação às estratégias de alocação de energia entre os indivíduos de cada sexo (Roff, 2002). Em virtude da seleção para maximizar a fecundidade e a sobrevivência da prole,

fêmeas alocam recursos e energia predominantemente no esforço somático, que afeta diretamente a longevidade e indiretamente a fecundidade, e no esforço parental, que beneficia a aptidão dos filhotes. Machos, por sua vez, investem em formas de maximizar o sucesso de acasalamento, o que leva a uma redução na alocação de recursos e energia para os esforços somático e parental (Roff, 2002).

Um exemplo bem estudado das diferenças entre machos e fêmeas em relação à alocação de recursos e energia, é o dimorfismo sexual em imunocompetência observado em muitas espécies de animais (Stoehr & Kokko, 2006). Esse dimorfismo sexual é entendido como resultado da demanda conflitante entre o esforço de acasalamento e a defesa imune. Dado o maior investimento dos machos no esforço de acasalamento, ocorre um comprometimento do esforço somático, em particular, da imunocompetência. No caso das fêmeas, um maior investimento em imunocompetência é favorecido, pois acarreta em maior longevidade e maior fecundidade (Roff, 2002). Apesar de toda atenção dedicada à imunocompetência nos últimos anos, vale destacar que ela é apenas um dos componentes do esforço somático. Pouca ou nenhuma atenção tem sido dispensada a outros componentes do esforço somático que podem aumentar a longevidade dos indivíduos, tais como defesas químicas. Assim como a maioria das respostas imunológicas de artrópodes, as defesas químicas são inespecíficas (Schmid-Hempel, 2005), ou seja, não são direcionadas a inimigos naturais em particular, porém envolvem gastos metabólicos sendo, portanto, custosas para os indivíduos (Bowers, 1992). Nesse contexto, seria esperado que o mesmo padrão de dimorfismo sexual em imunocompetência fosse encontrado também nas defesas químicas.

Um grupo de organismos adequado para estudos sobre custos e benefícios das defesas químicas são os opiliões, que constituem a terceira maior ordem em número de espécies dentro da classe Arachnida, com cerca de 6.500 espécies descritas (Machado *et al.*, 2007). Todas as espécies da ordem possuem um par de glândulas exócrinas localizadas nas margens anteriores do cefalotórax, perto da base da segundo par de pernas (Shultz, 1990). Essas glândulas são mais comumente conhecidas como glândulas odoríferas ou repugnatórias (Holmberg, 1983), pois liberam uma secreção de odor desagradável composta por uma grande variedade de compostos químicos, tais como fenóis, quinonas, alcoóis ou cetonas, dependendo do táxon em questão (revisão em Raspotnig *et al.*, 2015). A secreção liberada parece ser altamente eficaz contra a maioria dos inimigos naturais dos opiliões, incluindo vertebrados e invertebrados (e.g., Eisner *et al.*, 1971; 2004; Machado *et al.*, 2005). Embora a defesa química seja considerada o mecanismo de defesa mais eficaz em opiliões (Eisner *et al.*, 1978), esta parece ser empregada apenas quando todos os outros mecanismos

de defesa não obtiveram sucesso em impedir o ataque de predadores (Duffield *et al.*, 1981; Pomini *et al.*, 2010). Portanto, a produção dos compostos liberados pelas glândulas repugnatórias dos opiliões provavelmente é custosa e espera-se que sua emissão seja a última linha de defesa contra o ataque de um predador.

O primeiro trabalho sobre a natureza química da secreção repugnatória em opiliões foi realizado com *Acanthopachylus aculeatus* (Gonyleptidae), que libera benzoquinonas (Estable *et al.*, 1955). O composto liberado ficou conhecido como “gonyleptidina” e vários estudos subsequentes investigaram seus efeitos farmacológicos (Ardao & Freyre, 1956; Freyre *et al.*, 1958; Sáez & Drets, 1958). Somente no início do século XXI é que a eficiência da gonyleptidina contra predadores foi formalmente investigada (Eisner *et al.*, 2004). Atualmente, mais de 100 espécies de opiliões tiveram sua secreção defensiva caracterizada quimicamente (Raspotnig *et al.*, 2015), porém os trabalhos que testaram experimentalmente a função biológica desses compostos não ultrapassam uma dezena. Machado *et al.* (2002), por exemplo, demonstraram que a mistura de benzoquinonas liberada por *Serracutisoma gnaspinae* (Gonyleptidae) funciona como feromônio de alarme, induzindo a dispersão de indivíduos agregados. Pouco tempo depois, Eisner *et al.* (2004) e Machado *et al.* (2005) demonstraram que as benzoquinonas contidas na secreção repugnatória de *Acanthopachylus aculeatus* e *Acutisoma longipes* (Gonyleptidae), respectivamente, são potentes repelentes de formigas, aranhas e sapos. Apesar de lançarem luz sobre as possíveis funções biológicas dos compostos defensivos liberados pelos opiliões, todos os trabalhos publicados até o momento estão focados nos benefícios e nenhuma informação específica sobre custos foi explorada.

Recentemente, foi demonstrada experimentalmente a rota biossintética para a produção de benzoquinonas em opiliões da família Gonyleptidae (Rocha *et al.*, 2013). Adicionalmente, os autores também descobriram que os precursores dessas benzoquinonas são o acetato e o proprionato, duas moléculas amplamente utilizadas em rotas policetídicas em artrópodes (Morgan, 2004). A descoberta da rota biossintética para a produção de benzoquinonas em opiliões abre as portas para a investigação dos custos da produção de compostos defensivos no grupo. Uma vez que sabemos quais são os principais precursores, é possível explorar possíveis demandas conflitantes na alocação de recursos entre defesas e outras atividades, tal como a produção de ovos. Adicionalmente, é possível explorar os efeitos da quantidade e qualidade da dieta sobre a produção de benzoquinonas. Por fim, dada a enorme variação na intensidade do dimorfismo sexual em opiliões (Buzatto *et al.*, 2014), é possível explorar também se machos e fêmeas diferem na alocação de recursos para a produção de defesas químicas.

Esta tese tem como objetivo geral explorar os custos da produção de defesas químicas em opiliões e, para tanto, está dividida em três capítulos. No capítulo 1, investigamos como a produção de ovos pode comprometer a produção de defesas químicas e quais são as conseqüências da redução do volume de secreção liberada sobre a capacidade de defesa das fêmeas. No capítulo 2, investigamos como a quantidade e a qualidade da dieta influenciam a produção de defesas químicas. Por fim, o capítulo 3 teve como objetivos compreender as demandas conflitantes entre esforço de acasalamento (produção de armamentos e defesa territorial) e esforço somático (defesas químicas) entre machos e fêmeas.

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# Capítulo 1

## **Egg production constrains chemical defenses in a neotropical arachnid\***

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## **Abstract**

Female investment in large eggs increases the demand for fatty acids, which are allocated for yolk production. Since the biosynthetic pathway leading to fatty acids uses the same precursors used in the formation of polyketides, allocation trade-offs are expected to emerge. Therefore, egg production should constrain the investment in chemical defenses based on polyketides, such as benzoquinones. We tested this hypothesis using the harvestman *Acutiosoma longipes*, which produces large eggs and releases benzoquinones as chemical defense. We predicted that the amount of secretion released by ovigerous females (OFs) would be smaller than that of non-ovigerous females (NOF). We also conducted a series of bioassays in the field and in the laboratory to test whether egg production renders OFs more vulnerable to predation. OFs produce less secretion than NOFs, which is congruent with the hypothesis that egg production constrains the investment in chemical defenses. Results of the bioassays show that the secretion released by OFs is less effective in deterring potential predators (ants and spiders) than the secretion released by NOFs. In conclusion, females allocate resources to chemical defenses in a way that preserves a primary biological function related to reproduction. However, the trade-off between egg and secretion production makes OFs vulnerable to predators. We suggest that egg production is a critical moment in the life of harvestman females, representing perhaps the highest cost of reproduction in the group.

**Key-words:** benzoquinone, chemical ecology, chemical shield, costs of reproduction, maternal care, polyketides, resource allocation, trade-off

## Introduction

The production of large and heavily yolked eggs is perhaps the most widespread form of parental care among animals (Smiseth et al. 2012). Egg size is related to survival and growth of early hatched young in multiple taxa, including arthropods (Fox and Czesak 2000), fish (Morrongiello et al. 2012), amphibians (Dziminski et al. 2009), and birds (Williams 1994). Despite the benefits to the offspring, the production of large eggs may also impose costs to females because reproduction and self-maintenance are two processes that require great investment of energy and resources (review in Harshman and Zera 2007). Indeed, a negative correlation between survival and female investment in current reproduction is one of the most ubiquitous life-history trade-offs reported in the literature (Stearns 1992; Roff 2002). This pattern may emerge as a consequence of several different processes, but the allocation trade-off between reproduction and immune function has received the most attention in recent years (French et al. 2009; Moreno-García et al. 2013). Experimental evidence of insects, lizards, and birds have consistently shown that increases in the reproductive effort lead to decreases in the immune function and vice-versa (e.g., French et al. 2007; Knowles et al. 2009; Cox et al. 2010; Bascuñán-García et al. 2010; Stahlschmidt et al. 2013).

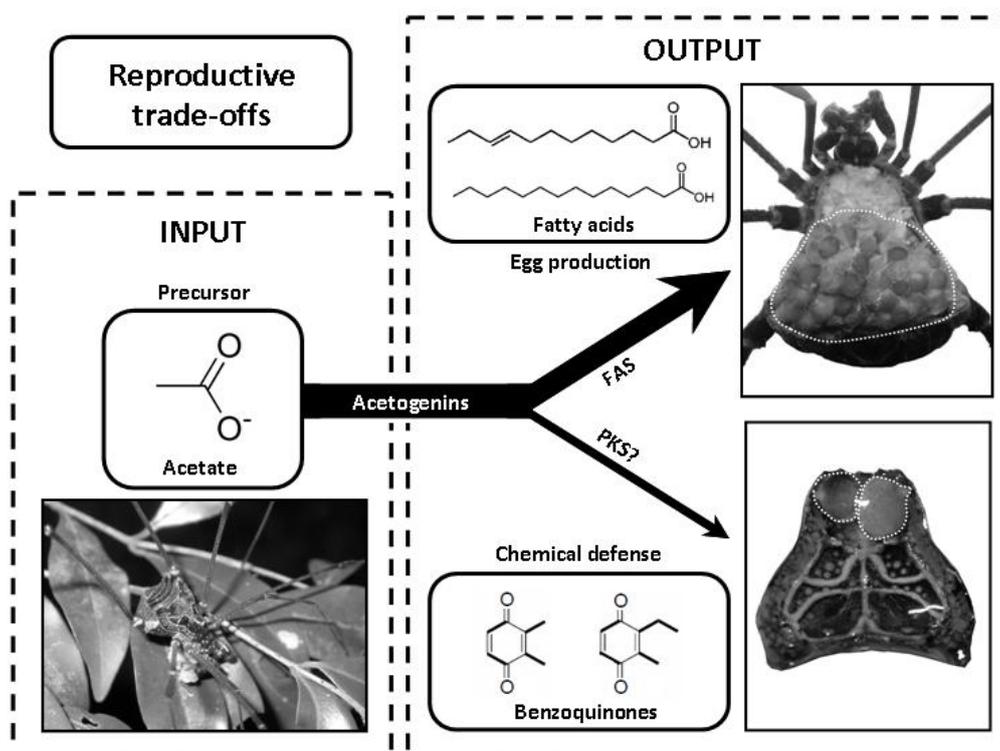
Although intensively studied, the immune system is one of the last lines of defense against natural enemies exhibited by animals (Schmid-Hempel 2005). Comparatively, few studies have investigated possible trade-offs between reproduction and other types of defense; most of them are focused on plants in which an increase in induced chemical defenses against herbivores promotes a decrease in fruit or seed production (Bazzaz et al. 1987; Stamp 2003; but see Neilson et al. 2013). There is also indirect evidence suggesting a trade-off between reproduction and chemical defenses among some marine animals. In the marine bryozoan *Membranipora membranacea*, for instance, colonies rapidly produce defensive spines in response to cues from a specialized predatory gastropod. In this case, colonies producing spines grow at lower rates than control colonies, and this growth decrease is directly translated into a reduced output of sexual propagules because fecundity is positively related to colony size in bryozoans (Harvell 1986). In the sponge *Oscarella balibaloï*, the production of secondary metabolites decreases during the period of embryogenesis, suggesting a trade-off between the resources dedicated to reproduction and the production of chemical defenses (Ivanisevic et al. 2011).

Given that chemical defenses are extremely common among many groups, and that there is strong evidence showing that these chemical defenses are costly (review in Berenbaum 1995), it is surprising that the trade-off between reproductive investment and the production of chemical weaponry has never been directly addressed in animals. Arthropods are perhaps one of the most tractable animal groups to explore this trade-off for several practical reasons: (a) many species are chemically defended (Eisner et al. 2005), (b) the composition and biosynthetic pathways of many chemical compounds are well-known (Morgan 2004), (c) female investment in egg size presents huge variation among taxa (Gilbert and Manica 2010), and (d) chemical defenses may be costly, and thus may compete for resources and energy with other life-history traits. Experimental evidence indicates that synthesizing chemical defenses can slow larval growth in holometabolous insects and also promote a reduction in the final size of the adults, which in turn may reduce their reproductive success (see Higginson et al. 2011 and references therein).

Quinones are polyketides found in the defensive glands of a wide range of arthropod species, including earwigs, cockroaches, termites, grasshoppers, beetles, millipedes, and harvestmen (Eisner et al. 2005). Despite the great diversity of quinonoid compounds produced by these arthropods, there are only two metabolic pathways for the generation of benzoquinones (Blum 1981). In millipedes (Diplopoda) and insects, benzoquinones may be biosynthesized from preformed aromatic rings of amino acids, such as tyrosine, or using acetate or propionate as precursors, suggesting a polyketide origin (Meinwald et al. 1966; Blum 1981; Morgan 2004; Pankewitz and Hilker 2008). In harvestmen (Opiliones), however, alkylated benzoquinones seem to be biosynthesized exclusively using acetate and propionate as precursors (Blum 1981; Rocha et al. 2013; Fig. 1).

According to the Y model of resource allocation, limited resources allocated to reproduction are not available for the soma (Harshman and Zera 2007). Under the perspective of an arthropod female, the investment in large and heavily yolked eggs increases the demand for fatty acids, which are allocated for the production of the vitelline membrane and lipid droplets imbedded in the yolk (Trogakos and Margaritis 2002; Fig. 1). Given that the biosynthetic pathways leading to long chain fatty acids are analogous to the formation of polyketides, and that the same precursors are used by both fatty acid synthases and polyketide synthases, allocation trade-offs are expected to emerge (Pankewitz and Hilker 2008; Fig. 1). Therefore, egg production should constrain the investment in chemical defenses based on polyketide compounds, such as alkylated benzoquinones. We tested this hypothesis using the harvestman *Acutiosoma longipes* (Gonyleptidae) as study organism

(more details in ‘Study species’ below). Our prediction was that the amount of secretion stored in the glandular sac of ovigerous females would be smaller than the amount stored by non-ovigerous females. We also conducted a series of bioassays in the field and in the laboratory to test whether egg production renders ovigerous females more vulnerable to predation. Our predictions were: (i) the amount of secretion released by ovigerous females would be less effective in deterring potential predators than the amount released by non-ovigerous females, and (ii) the chemical shield provided by the defensive secretion (Machado et al. 2005) would last longer in the non-ovigerous than in the ovigerous females.



**Fig. 1.** Schematic representation of the Y model of resource allocation, which proposes that limited resources allocated to reproduction are not available for the rest of the body. In our study, resource input is shown at the left box. Acetate, which is an important precursor of many organic molecules, is acquired when harvestman females feed on live and dead arthropods, fungi, and fruits. The output is shown in the right box illustrating the trade-off between egg production and chemical defenses. The investment in large and heavily yolked eggs in harvestmen (indicated by the white dotted line in the upper photo) increases the demand for fatty acids, which are allocated for the production of the vitelline membrane and lipid droplets imbedded in the yolk. The biosynthetic pathway leading to long chain fatty acids is analogous to the formation of polyketides, and the same precursor (acetate) may be used by both fatty acid synthases (FAS) and polyketide synthases (PKS). Benzoquinones are repellent polyketides produced by many harvestman species in a pair of exocrine glands located at the anterior margins of the carapace (indicated by the white dotted line in the lower photo). As a consequence of allocation trade-offs, the investment in chemical defenses based on benzoquinones should be constrained by egg production.

## Methods

### *Study species*

Individuals of *A. longipes* produce a large amount of defensive secretion composed of two alkylated 1,4-benzoquinones that are released through a pair of exocrine glands located at the anterior margins of the carapace (Machado et al. 2005; Fig. 1). Although these benzoquinones are highly effective in repelling several invertebrate and vertebrate predators, they are employed only when all other evasive measures were unsuccessful in preventing the predator attack (Machado et al. 2000, 2005), which suggests that their production is costly. Females also produce 80-200 large yolked eggs that occupy more than 50% of their body volume before oviposition (Machado and Oliveira 1998; Machado and Macías-Ordóñez 2007; Fig. 1). These eggs are laid on rock walls inside caves and are guarded by the mother until hatching and dispersal of the nymphs (Machado and Oliveira 1998).

### *Collection of individuals*

We collected individuals of *A. longipes* inside caves at Parque Florestal do Itapetinga (23°15' S; 46°45' W), Atibaia, state of São Paulo, southeastern Brazil, between October 2003 and May 2004 (COTEC permission # 41.852/2001). We selected adult females in three phases of their reproductive cycle. (1) Non-ovigerous females (NOFs) were those bearing no egg and that were not guarding eggs. (2) Ovigerous females (OFs) were those bearing mature eggs and that were about to oviposit (Fig. 1). These females can be easily recognized because they show free tergites spaced out with the intersegmental membrane clearly visible (Machado and Macías-Ordóñez 2007). (3) Egg-guarding females were those that already oviposited and were guarding their eggs for nearly 15 days. The age of a clutch in harvestman can be easily inferred because eggs change in color and size over the course of the embryonic development (Gnaspini 2007). We selected 15 day-old clutches because previous laboratory experiments with other quinone-releasing harvestman species indicate that it is the time requested for starved individuals to recover most of their gland volume (Nazareth et al. unpublished data). In the laboratory, we placed females belonging to each reproductive phase in different terraria (60 x 40 cm base, 35 cm high) containing pieces of cotton wetted with water to maintain the humidity.

To exclude the possibility that the concentration of the secretion differs between females in different reproductive phases, we sampled additional ovigerous and non-ovigerous females in the same locality in May 2014. We did not sample egg-guarding females because our previous collection indicated that there is no difference in the mass of secretion produced by females in this phase and non-ovigerous females (see Results).

#### *Production of chemical defenses*

We quantified the mass of defensive secretion released by females in each reproductive phase 24 h after collection in the field. First, we weighed a small piece of cotton wool, seized an individual by hand, and induced the emission of exudate by pressing the cotton wool held by tweezers against the gland openings. We repeated this procedure three times to ensure that the gland sacs were completely depleted, and then weighed the cotton wool again. Since harvestmen usually release water from the mouth before or after the emission of defensive secretion (Gnaspini 2007), we blocked the mouthparts of the females with another piece of cotton wool when milking them of secretion to avoid that enteric water fluid would mix with the gland exudate. We discarded this piece of cotton wool soaked with water, and used the difference in weigh between the second and the first measurements of the cotton wool soaked with exudate to estimate the mass of secretion released by each female. Finally, we measured the dorsal scute length (DSL) of each female using digital calipers (to nearest 0.01 mm). DSL is a standard estimate of body size in harvestmen because it does not change according to hunger or reproductive phase (Requena et al. 2012).

To quantify the concentration of benzoquinones released by OFs and NOFs from the second sample, we induced the emission of exudate as described above. We then washed the cotton wool tree times with 500  $\mu\text{L}$  of  $\text{CH}_2\text{Cl}_2$  to guarantee complete extraction of benzoquinones. We added 1  $\mu\text{L}$  of the obtained solution (solvent and secretion) to 600  $\mu\text{L}$  of  $\text{CH}_2\text{Cl}_2$  and 1  $\mu\text{L}$  of benzophenona (internal standard), and analyzed the sample by gas chromatography. We used the Shimadzu GC-FID 2014 gas chromatograph coupled with an AOC20i autosampler fitted with a RTX-5 capillary column. The oven temperature was as follow: 40  $^\circ\text{C}$  (2 min), 5  $^\circ\text{C min}^{-1}$  to 200  $^\circ\text{C}$  (4 min). Nitrogen was used as the carrier gas at a linear velocity, column flow, and purge of 18.7  $\text{cm s}^{-1}$ , 1  $\text{mL min}^{-1}$  and 3  $\text{mL min}^{-1}$ , respectively. Injections of 1  $\mu\text{L}$  were carried out in a splitless mode, during 1 min at 220  $^\circ\text{C}$  and 32.5 kPa. Temperature, air (20%  $\text{O}_2$  in  $\text{N}_2$ ), and hydrogen flows of detector were set at 250  $^\circ\text{C}$ , 400 and 40  $\text{ml min}^{-1}$ , respectively. We calculated the relative amount of the two benzoquinones contained in each sample by the ratio between the area and mass of internal

standard and the area of identified benzoquinones in each sample. We estimated total benzoquinones per sample as the sum of the net quantities of the two benzoquinones present in the mixture released by *A. longipes* females. We quantified benzoquinones from the linear regression equation ( $R^2=0.994$ ) of a calibration curve constructed for 1,4-benzoquinone, so that all amounts are expressed as 1,4- benzoquinone equivalents.

We used general linear models (GLMs) to compare the mass of secretion and concentration of benzoquinones released by females (response variables with Gaussian error distribution) in different reproductive phases (categorical predictor variable), controlling for the effect of body size (continuous predictor variable). Given that we did not expect any interaction between reproductive phase and body size, our models include only the additive effect of these variables (exploratory analyses showed that the interaction is indeed not significant – data not shown).

### *Bioassays*

We conducted a series of bioassays to test the efficiency of NOF and OF secretions against two groups of potential predators: ants and spiders. Immediately before each trial, we milked a female of *A. longipes* of secretion by seizing it by hand and collecting the exudate with capillary tubes. We never repeated the same female in different trials because repeated milking of the same individual reduces the amount of released secretion.

### *Tests with ants*

We conducted two bioassays to test the potential effect of the secretion on ants: one in the field to test the repellent potential of NOF and OF secretions, and another in the laboratory to test the effectiveness of NOF and OF secretions as a chemical shield. The field experiment consisted of presenting baits made up of pieces of filter paper (1 cm<sup>2</sup>) embedded with a saturated sugar solution and placed on plastic dishes (5 cm diameter). We randomly distributed 100 baits on the forest floor (5 m from each other) at Parque Florestal do Itapetinga. When ants were feeding at the margin of the bait, we stimulated them discharging a solution in the center of the filter paper with a syringe. The constitution of this solution differed among three experimental groups: (1) glandular secretion of one NOF diluted in 100 µL of water, (2) glandular secretion of one OF diluted in 100 µL of water, and (3) 100 µL of distilled water (control). In the three experimental groups, we counted the number of ants in contact with the bait before and 5 s after presentation of solution.

To analyze the data, we created a repellency index ( $RI$ ) given by  $RI = \frac{N_0 - N_5}{N_0}$ , where  $N_0$  is the number of ants in contact with the bait before and  $N_5$  is the number of ants 5 s after presentation of solution. The  $RI$  indicates the proportion of ants that were repelled from the sugar bait after stimulation, so that when  $RI = 0$  no ant was repelled and when  $RI = 1$  all ants were repelled from the bait. We performed a GLM on the repellency index (response variable with Gaussian error distribution) including ant species and the experimental groups as predictor categorical variables. Given that the response of the workers to the different experimental groups could vary according to the ant species, we included the interaction between these two variables in the model. If egg production constrains the production of chemical defenses, OF secretion should be less efficient in repelling ants. Thus, the reduction in the number of ants in contact with the baits after stimulation with OF secretion should be intermediate between the control group and those stimulated with NOF secretion.

In the laboratory experiment, we used five colonies of large predatory ants that occur synoptically with *A. longipes*: two of *Odontomachus chelifer* and three of *Pachycondyla striata* (both Ponerinae). We collected all colonies in the field and brought them to the laboratory, where they were placed inside plastic trays (25 x 40 cm). In each tray, we placed two test tubes (2 cm diameter x 15 cm length) containing water trapped behind a cotton plug that were used as nests by the ants. Nearly two months after the colonies were brought to the laboratory, we conducted the experiment of chemical shield. Inside each tray, we presented a glass cover slip (1 x 10 cm) divided in three equal parts randomly designated as treatment 1, treatment 2, and control. Treatments 1 and 2 consisted of a filter paper (1 cm<sup>2</sup>) wetted with 200 µL of a saturated sugar solution mixed with the glandular secretion of one NOF and one OF, respectively. The control contained only a filter paper (1 cm<sup>2</sup>) wetted with 200 µL of a saturated sugar solution. We counted the total number of ants feeding on each bait at 2 min-intervals during 40 min after the first contact.

To analyze the data, we performed repeated measures analysis of variance using the number of ants feeding on the baits at each 2 min-interval as response variable and experimental groups as predictors. Degrees of freedom were corrected using the Greenhouse-Geisser procedure to avoid sphericity problems. Given that the number of colonies of each ant species was limited, we did not include species identity in the analysis. If egg production constrains the production of chemical defenses, the chemical shield promoted by OF secretion should last less time than that promoted by NOF secretion for both ant species.

### *Test with spiders*

*Trechaleoides biocellatus* (Trechaleidae) is a large (2 - 3 cm body length) wandering spider, abundant in the study site, which is generally found near river margins or in other moist habitats, such as caves (Machado et al. 2005). We collected individuals of the species at Parque Florestal do Itapetinga between October 2003 and May 2004, and maintained them in individual cages (20 x 10 cm base, 15 cm high) containing a piece of cotton wetted with water to maintain the humidity. Only subadults and adults of both sexes ( $n = 60$ ) were used in the experiments, and each individual was starved for 5-6 days before the experiments to bring them to a similar level of hunger.

To test the role of the defensive secretion alone and exclude the interference of other possible defenses, such as spines on legs and pedipalps, we did not offer individuals of *A. longipes* directly to the spiders (following Machado et al. 2005). Rather, we offered individuals of the common cricket *Gryllus gryllus* (nearly 1 cm of body length), which the spiders promptly took as prey. In order to ensure that the crickets were unable to promote injuries to the spiders, we removed the hind legs (armed with several spines) of the crickets just before the experiment. After the cricket was grabbed, we stimulated each spider with one of the following solutions: 1) OF secretion diluted in 100  $\mu$ L of water ( $n = 20$ ); 2) NOF secretion diluted in 100  $\mu$ L of water ( $n = 20$ ), or 3) 100  $\mu$ L of distilled water (control). We applied the solutions with a syringe directly to the base of the chelicerae. Spiders that extricated the chelicerae and abandoned the prey within 5 min were scored as respondents (following Eisner et al. 1997). We compared the number of spiders that released or not the prey using two Fisher exact tests: one between control and OF secretion, and other between NOF and OF secretions. Given that we performed two analyses using the same dataset, we used the Bonferroni correction to adjust the p values. If egg production constrains the production of chemical defenses, OF secretion should be less efficient in repelling spiders. Thus, the number of crickets released by the spiders stimulated with OF secretion should be lower when compared to the spiders stimulated with NOF secretion.

## **Results**

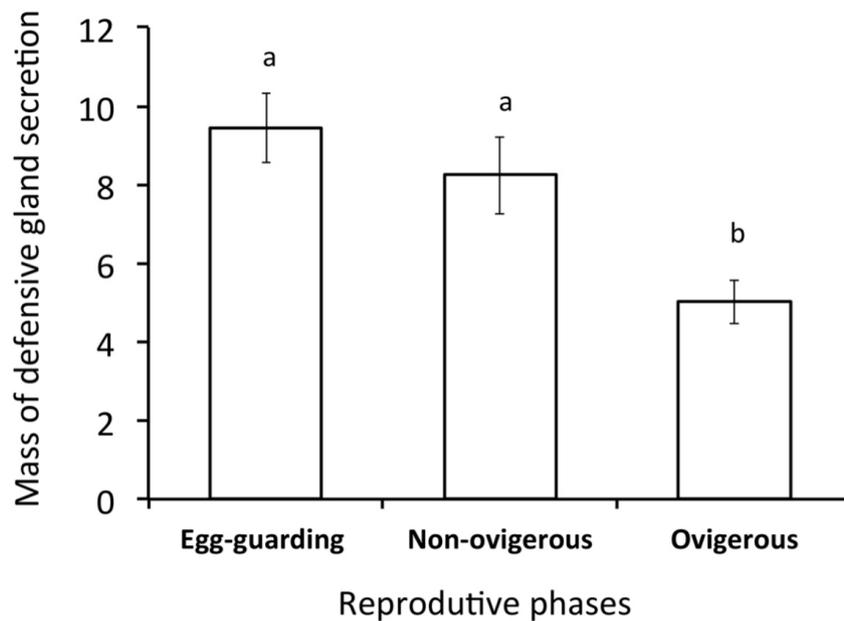
### *Production of chemical defenses*

The mass of defensive secretion released by the females was affected by their reproductive phase, but not by their body size. Egg-guarding females ( $n = 12$ ) and NOFs ( $n = 25$ ) released a similar mass of secretion, which was on average 71.8% greater than the mass of

secretion released by OFs ( $n = 25$ ; Fig. 2; Table 1). Females produced defensive secretion with similar same concentration of benzoquinones, regardless of body size and reproductive stage (mean  $\pm$  SD): NOFs =  $21.03 \pm 15.28 \mu\text{g/mL}$  and OFs =  $18.97 \pm 12.93 \mu\text{g/mL}$  (Table 1).

**Table 1.** Results of the GLM testing the effect of body size and reproductive phase on the mass of defensive gland secretion and concentration of total benzoquinones released by females of the harvestman *Acutisoma longipes*. Significant p-values are shown in bold.

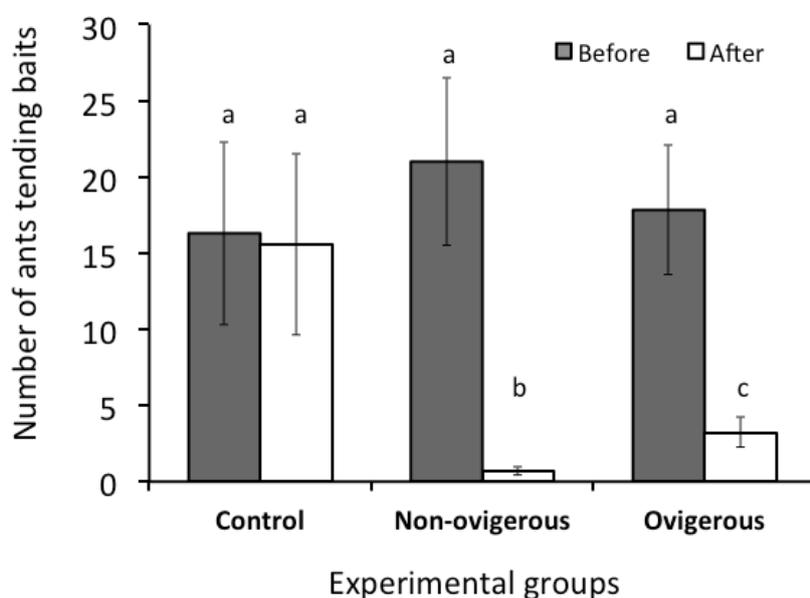
Effect	DF	MS	F	p
<b>Mass of defensive secretion</b>				
Body size	1	35.507	2.430	0.125
Reproductive phase (egg-guarding, non-ovigerous, and ovigerous females)	2	93.733	6.416	<b>0.003</b>
Error	58	14.611		
<b>Concentration of total benzoquinones</b>				
Body size	1	0.040	0.315	0.576
Reproductive phase (non-ovigerous and ovigerous females)	1	0.168	1.296	0.258
Error	83	0.128		



**Fig. 2.** Mass of defensive secretion (mean  $\pm$  SE) released by egg-guarding, non-ovigerous, and ovigerous females of the harvestman *Acutisoma longipes*. Different letters indicate significant differences (post-hoc test,  $p < 0.05$ ).

### Tests with ants

In total, 51 baits were visited by workers of seven ant species in the field: three by *Crematogaster* sp. (one for each experimental group), three by *Pachycondyla striata* (one for each experimental group), three by *Pheidole* sp.1 (one for each experimental group), six by *Odontomachus chelifer* (two for each experimental group), nine by *Camponotus* sp. (three for each experimental group), nine by *Pheidole* sp.2 (three for each experimental group), and 18 by *Gnamptogenys* sp. (six for each experimental group). The discharge of defensive secretions induced a marked reduction in the number of ants feeding on the sugar baits, regardless of the ant species (Table 2). Baits treated with NOF secretion, however, had slightly higher repellency than those treated with OF secretion (Fig. 3). No significant reduction was observed in the control baits (Fig. 3).



**Fig. 3.** Results of the field experiment in which the number of ants tending sugar baits was counted before and after stimulation with one of the three experimental groups: (1) secretion of one non-ovigerous female of *Acutisoma longipes*, (2) secretion of one ovigerous female of *A. longipes*, and (3) distilled water (control). Different letters indicate significant differences (post-hoc test,  $p < 0.05$ ).

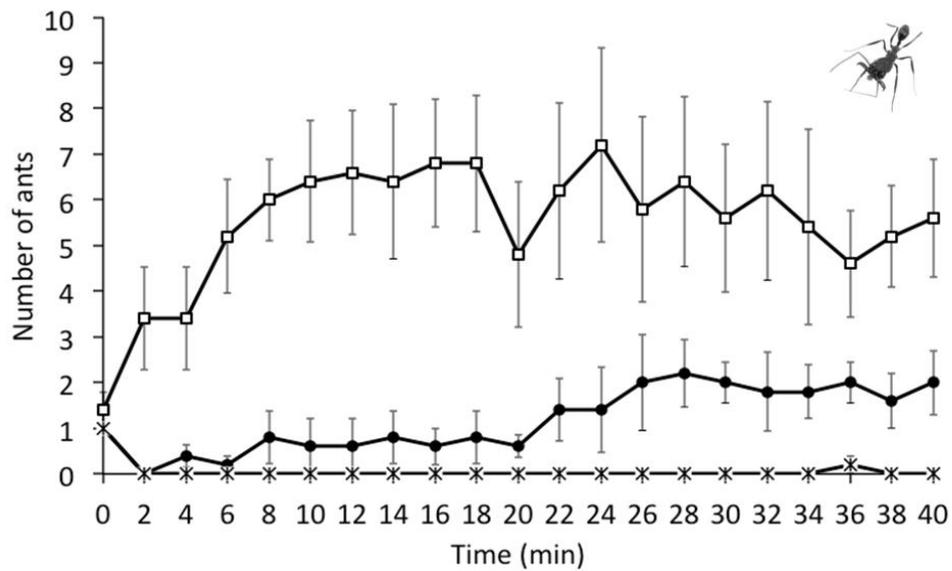
**Table 2.** Results of the GLM testing the effect of ant species and experimental groups on the repellency index. The experimental groups were: (1) secretion of one non-ovigerous female of *Acutisoma longipes*, (2) secretion of one ovigerous female of *A. longipes*, and (3) distilled water (control). Significant p-values are shown in bold.

Effect	DF	MS	F	P
Intercept	1	13.109	601.975	<b>&lt; 0.001</b>
Ant species	6	0.013	0.609	0.721
Experimental group	2	2.664	122.322	<b>&lt; 0.001</b>
Ant species x Experimental group	12	0.014	0.661	0.773
Error	30	0.022		

In the laboratory bioassay, the number of workers tending control baits increased fast from 0 to 12 min. After this period until the end of the experiment, we observed an average of five to seven workers tending the baits (Fig. 4). No ant was observed tending the baits containing OF secretion during the first 2 min. After 4 min, however, the number of workers tending the baits increased slowly, and from 22 min until the end of the experiment, we observed an average one to two workers tending the baits (Fig. 4). No ant was observed tending the baits containing NOF secretion during the entire experiment in almost all colonies (Fig. 4). In general, there was a significant interaction between time and experimental group ( $F_{40, 160} = 4.55$ ;  $p = 0.002$ ).

#### *Tests with spiders*

Only one individual of *T. biocellatus* released the prey in the control group ( $n = 20$ ). When individuals were stimulated with OF secretion ( $n = 20$ ), only two released the prey and this frequency does not differ from the control (Fisher exact test,  $p = 1.00$ ). When individuals were stimulated with NOF secretion ( $n = 20$ ), nine released the prey and this frequency is significantly higher than the group stimulated with OF secretion (Fisher exact test,  $p = 0.016$ ).



**Fig. 4.** Mean ( $\pm$  SE) number of workers of the ants *Odontomachus chelifer* and *Pachycondyla striata* tending three types of baits: (1) sugar solution + secretion of one non-ovigerous female of *Acutisoma longipes*, (2) sugar solution + secretion of one ovigerous female of *A. longipes*, and (3) sugar solution (control). The photo illustrates the experimental setup of an *O. chelifer* colony at 14 min of experiment.

## Discussion

We found that females of the harvestman *A. longipes* bearing mature eggs produce less defensive secretion than females in other reproductive phases, including non-ovigerous and egg-guarding females. Moreover, we found no difference in the concentration of total benzoquinones released by OFs and NOFs, indicating that there is no compensation related to increased concentration of defensive compounds during egg production. These results are congruent with the hypothesis that egg production constrains the investment in chemical defenses based on benzoquinones (Fig. 1). Finally, the results of our bioassays clearly

indicate that the low amount of secretion released by OFs is less effective in deterring potential predators (ants and spiders) than the high amount released by NOF. In what follows, we will explore these results in more detail and discuss their implications for our understanding on the costs of reproduction in a chemically defended animal.

During egg production, a large amount of yolk must be stored in the oocytes in a relatively short period of time to provide nutritional supply for the developing embryo (Bownes 1986). The increased physiological requirement promoted by egg production leads females of many species to intensify their foraging activities (Helfman 1990). In the fishing spider *Dolomedes triton*, for instance, females switch from a sit-and-wait strategy to more active foraging upon maturation (Kreiter and Wise 1996, 2001). In the harvestman *Serracutisoma spelaeum*, OFs forage more frequently than NOFs, leaving the cave habitat to search for food almost every night (Gnaspini 1996). The same pattern seems to occur with *A. longipes* (G. Machado, pers. obs.), and our results suggest that the resources acquired by OFs during this period of intense foraging activity are invested predominantly in egg production, rather than chemical defenses. Histological studies of *A. longipes* support this suggestion, indicating that during the period of egg production there is a marked increase in the lipid content of the fat body (Tomaino-Gomes 2008), an organ that is the source of most part of the yolk received by the ovarian follicles (Trogakos and Margaritis 2002).

The possible trade-off reported here for *A. longipes* contrasts with the results obtained in a previous study with *Zophobas atratus*, a tenebrionid beetle that produces two alkylated benzoquinones also found in many harvestman species (Rocha et al. 2013). In this beetle, egg production did not affect the investment in chemical defenses, so that mated females that produced twice as many eggs as virgin females released nearly the same amount of defensive secretions (Hill and Tschinkel 1985). The authors suggest that individuals do not channel much energy into the production of defensive secretions, and that a marked trade-off between egg and secretion production should be found in species that invest more energy in chemical defenses (Hill and Tschinkel 1985). We think, however, that the lack of pattern reported for *Z. atratus* is related to the high abundance of food provided to the beetles in the laboratory. Trade-offs are more likely to emerge when internal energy reserves are limited (Harshman and Zera 2007), and under natural conditions, egg-producing *A. longipes* females are probably food limited because most foraging trips outside the cave habitat are unsuccessful (Machado et al. 2000). This may explain why we detected a negative influence of egg production on the total mass of secretion produced by OF.

Egg-guarding females released the same mass of secretion reported for NOFs (Fig. 2), indicating that the investment in chemical defenses is resumed after oviposition. Given that females are prevented from foraging while caring for the offspring, and remain stationary on the clutch all day long during the entire period of embryonic development (Machado and Oliveira 1998), which resources are used to produce benzoquinones? Detailed histological studies of *A. longipes* show that some oocytes are reabsorbed during the period of maternal care (Tomaino-Gomes 2008), and we suggest that the nutrients obtained from the oocytes are the source for the production of benzoquinones during the caring period. The possible translocation of resources between the endpoints of the Y model of resource allocation reinforces the notion that the same precursors can be used to produce both fatty acids and polyketides (Pankewitz and Hilker 2008), giving rise to the trade-off we are proposing here between egg and benzoquinone production (Fig. 1). The increased production of chemical defenses after oviposition may be particularly important for egg-guarding females, which remain on the clutch for more than one month probably exposed to active-hunting predators (Machado and Oliveira 1998). In fact, a long-term field experiment with the harvestman *S. proximum*, which is closely related to *A. longipes*, indicates that the mortality of egg-guarding females is not reduced when compared with females prevented from caring (Buzatto et al. 2007), suggesting that females are well-protected against predation during the period of parental care.

The strong irritating properties of benzoquinones are known to repel numerous invertebrate and vertebrate predators (see Eisner et al. 2005 and references therein). In a previous study with *A. longipes*, the defensive secretion of adult males and NOFs repelled seven ant species, two species of large wandering spiders, and one frog species (Machado et al. 2005). Using similar protocols, we showed here that the efficiency of the defensive gland secretion released by OFs is reduced when compared with NOFs. In the field experiment with ants, the repellency index of OF secretion was slightly lower than that of NOF (Fig. 3). However, the variance in the repellency index of the secretion released by OFs was much higher, with some values overlapping the values of the control group (Fig. 3). In the laboratory experiment, the chemical shield promoted by the OF secretion lasted considerably less time than that of NOF secretion (Fig. 4). After 40 min, an average of two ants was tending the baits wetted with OF secretion while no ant was tending the baits wetted with NOF secretion (Fig. 4). Finally, spiders stimulated with OF secretion released the crickets 4.5 times less frequently than those stimulated with NOF secretion.

Field studies with several arthropod species, including three neotropical harvestmen, indicate that individuals that are more active during the reproductive period are more frequently captured by ambush predators than sedentary individuals (McCauley and Lawson 1986; Polis et al. 1998; Buzatto et al. 2011; Requena et al. 2012; Requena and Machado 2015). As we mentioned above, females of goniosomatine harvestmen increase their foraging activities during the period of egg production and leave the cave habitat on a daily basis, while males and NOFs may remain stationary inside the cave for three or more days (Gnaspini 1996; Machado et al. 2000). Therefore, highly vagile OFs are probably under a higher risk of predation than other conspecifics, especially if the egg load also decreases female locomotor ability, as it has already been reported for females of many animal groups (review in Magnhagen 1991). Additionally, the results of our bioassays suggest that the trade-off between the egg and benzoquinone production makes OFs particularly vulnerable to predation when compared with NOF. Taken together, these findings support the notion that egg production is a critical moment in the life of harvestman females, representing perhaps the highest cost of reproduction, as also suggested for many bird species (Williams 2005).

In conclusion, females allocate resources to chemical defenses in a way that preserves a primary biological function related to reproduction. As far as we know, this is the first time this trade-off has been directly demonstrated for animals. In the future, mark-recapture studies should be conducted in the field to assess whether mortality rates of OFs are higher than NOFs. Moreover, it would be interesting to investigate whether OFs fed *ad libitum* in the laboratory are able to channel more resources to egg production so that the trade-off observed under field conditions is somehow attenuated. Finally, a metabolomic approach to the trade-off between egg and benzoquinone production could be valuable to characterize the physiological responses of the females at the biochemical level (see Bundy et al. 2009).

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# Capítulo 2

## **Chemical defense as a condition-dependent trait in harvestmen (Arachnida: Opiliones)\***

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## **Abstract**

The expression of costly fitness-related traits, such as exaggerated secondary sexual traits and immune response, is usually dependent on the amount of food available to individuals. Considering that chemical defenses are also a costly fitness-related trait, the production of deterrent compounds should be condition-dependent. Here we experimentally tested how food availability and diet composition affect the production of benzoquinones by the harvestman *Magnispina neptunus* (Gonyleptidae). We hypothesize that: (1) the production of chemical defenses is condition-dependent and (2) the chemical composition of the food influences the production of chemical defenses. We manipulated the diet of the individuals in the laboratory and created four experimental groups: well nourished with acetate (the precursor of benzoquinones), well nourished without acetate, poorly nourished with acetate, and poorly nourished without acetate. Regardless of the sex, well nourished individuals produced secretions with greater mass and higher concentration of benzoquinones than poorly fed individuals. We detected no significant effect of diet composition or its interaction with food availability on the mass or concentration of secretion. In conclusion, the production of benzoquinones by the harvestman *M. neptunus* is clearly condition-dependent. Therefore, food deprivation, caused either by periods of prey shortage or by foraging limitation associated to time consuming activities such as parental care, may compromise the efficiency of chemical defenses and make individuals more exposed to predators.

**Key Words:** acetate, benzoquinone, costs, diet composition, food availability, parental care.

## Introduction

Condition is the result of the amount of food available to an individual, which is the environmental component of condition, and also the efficiency with which these resources are converted into fitness, which is the genetic component of condition (Rowe and Houle 1996). Although genetic variation in condition remains poorly understood, numerous experimental studies have demonstrated that the expression of costly fitness-related traits is highly dependent on the amount of food available to the individuals. For instance, individuals that have access to more nutritional resources usually exhibit more exaggerated secondary sexual traits (Houslay and Bussière 2012) or more effective immune responses (Zuk and Stoehr 2002). Considering that chemical defenses are also a costly fitness-related trait (Bowers 1992), the production of deterrent compounds should also be condition-dependent. The connection between nutritional resources and chemical defenses is straightforward in species that sequester deterrent compounds from their host plants or their animal prey (Jones et al. 1987; Calcagno et al. 2004). In these species, individuals consuming food that does not provide deterrent compounds may be able to keep proper body condition, but are unable to replenish their chemical defenses (Nishida 2002; Saporito et al. 2012). To our knowledge, however, there are only few studies investigating whether the amount of chemical defenses is dependent on the quantity or quality of resources in species that need to pay the costs of producing their own deterrent compounds (e.g., Kögel et al. 2012).

Numerous arthropod species produce chemical compounds that discourage predators and increase their probability of escaping an attack (Eisner et al. 2005). While chemicals are probably one of the most effective defense mechanisms against predators, they must be replenished frequently (Blum 1981; Whitman et al. 1994). Therefore, only individuals in good condition should be able to afford the costs of constant production of deterrent compounds. A suitable group of arthropods to test this hypothesis are arachnids of the order Opiliones, commonly known as harvestmen or daddy longlegs. Harvestmen have a pair of large exocrine glands located at the anterior margins of the carapace that release deterrent compounds (Gnaspini and Hara 2007). The chemical identity of these compounds varies both within and between families, but a great number of species belonging to the superfamily Gonyleptoidea releases alkylated benzoquinones (Raspotnig et al. 2015), which are highly effective repellents of ants, spiders, and ectothermic vertebrates, such as frogs and lizards (Duffield et al. 1981; Eisner et al. 2004; Machado et al. 2005).

The alkylated benzoquinones produced by harvestmen are biosynthesized using acetate and propionate as precursors (Rocha et al. 2013), which are also import precursors of many organic molecules, including fatty acids (Pankewitz and Hilker 2008). In females, fatty acids are allocated for the production of the vitelline membrane and lipid droplets imbedded in the yolk (Trouwakos and Margaritis 2002). Given that the same precursors, acquired when females feed on a great variety of food items, are used to produce long chain fatty acids and benzoquinones, allocation trade-offs are expected to emerge. In a recent study, we indeed showed that harvestman females bearing mature eggs produce less benzoquinones than females in other reproductive phases (Nazareth and Machado 2015). This result reinforces the notion that chemical defenses in harvestmen are costly, and suggests that food containing great amounts of the benzoquinone precursors should improve the production of chemical defenses by attenuating allocation trade-offs.

Here we experimentally tested how food availability and diet composition affect the production of benzoquinones by the harvestman *Magnispina neptunus* (Gonyleptidae). Individuals of this species produce a defensive secretion composed of two benzoquinones: 2-methyl-1,4-benzoquinone and 2-ethyl-1,4-benzoquinone (Rocha et al. 2013). We hypothesize that the production of chemical defenses is condition-dependent and predict that (1) well nourished individuals will produce more defensive secretions than poorly nourished individuals. We also hypothesize that the chemical composition of the food has an important role in the production of chemical defenses and predict that (2) individuals receiving benzoquinone precursors (acetate) in the diet will produce more defensive secretions than individuals deprived from the precursors because the building blocks of the chemical deterrents are promptly available to be used by the former, but not the latter.

## Methods and Materials

### *Collection of individuals and maintenance*

We collected 120 individuals (60 males and 60 females) of *M. neptunus* in August 2014 in the municipality of Arraial d'Ajuda, state of Bahia, northeastern Brazil (SISBIO permit number 38607-1). Two days later we took all individuals to the laboratory where we started collecting data. In the laboratory, we kept the harvestmen individually in small Petri dishes (diameter 8.5 cm) containing a piece of wet cotton and a dry leaf as shelter. The conditions in the laboratory were: mean ( $\pm$ SD) temperature =  $24 \pm 6$  °C (mean  $\pm$  SD), mean ( $\pm$ SD) relative humidity =  $70 \pm 18\%$ , and photoperiod of 12 hours of light and 12 hours of darkness.

### *Initial quantification of defensive secretions*

Before starting the experiment, we emptied the glands of all individuals. In order to do that, we first weighed a small piece of sterile cotton, seized each individual by hand, and induced the emission of defensive secretion by pressing the piece of cotton held against its gland openings. We repeated this procedure 2-3 times to ensure that the glands were completely depleted, and then weighed the cotton soaked with secretion. We calculated the mass of defensive secretion as the difference in cotton weight after and before the extraction.

To calculate the concentration of benzoquinones released by the individuals, we washed the cotton soaked with secretion with 500  $\mu\text{L}$  of  $\text{CH}_2\text{Cl}_2$ . This procedure was repeated twice to guarantee the complete extraction of benzoquinones. We then added 1  $\mu\text{L}$  of benzophenona as an internal standard, and injected the resulting solution into a Shimadzu GC-FID 2014 gas chromatograph coupled with an AOC20i autosampler fitted with a RTX-5 capillary column. The oven temperature was as follow: 40  $^\circ\text{C}$  (2 min), 5  $^\circ\text{C min}^{-1}$  to 200  $^\circ\text{C}$  (4 min). Nitrogen was used as the carrier gas at a linear velocity, column flow, and purge of 18.7  $\text{cm s}^{-1}$ , 1  $\text{mL min}^{-1}$  and 3  $\text{mL min}^{-1}$ , respectively. Injections of 1  $\mu\text{l}$  were carried out in a splitless mode, during 1 min at 220  $^\circ\text{C}$  and 32.5 kPa. Temperature, air (20%  $\text{O}_2$  in  $\text{N}_2$ ), and hydrogen flows of detector were set at 250  $^\circ\text{C}$ , 400 and 40  $\text{mL min}^{-1}$ , respectively.

From the chromatogram, we calculated the relative amount of the two benzoquinones in each sample by the ratio between the area and mass of the internal standard and the area of the benzoquinones in each sample. We estimated total benzoquinones per sample as the sum of the net quantities of the two benzoquinones present in the mixture released by each individual in each extraction. Finally, we quantified benzoquinones from the linear regression equation ( $R^2=0.999$ ) of a calibration curve constructed for 1,4-benzoquinone, so that all amounts presented here are expressed as 1,4-benzoquinone equivalents (according to Nazareth and Machado 2015).

### *Experimental setup*

Once the defensive secretion was extracted, we randomly divided the individuals into four experimental groups with 15 males and 15 females each. Individuals of each experimental group were fed during 20 days with a different frequency and diet composition: (1) well nourished without acetate (WNA<sup>-</sup>): individuals fed every two days with a diet consisting of 60 mg protein (97%) diluted in a drop of water; (2) well nourished with acetate (WNA<sup>+</sup>): individuals fed every two days with a diet consisting of two parts of

protein (97%) and one part of pure acetate (40 mg protein + 20 mg acetate) diluted in a drop of water; (3) poorly nourished without acetate (PNA<sup>-</sup>): individuals fed once every six days with the same diet as group WNA<sup>-</sup>; and (4) poorly nourished with acetate (PNA<sup>+</sup>): individuals fed once every six days with the same diet as group WNA<sup>+</sup>. During the 20 day-period of diet manipulation, individuals of groups (1) and (2) received 600 mg of food, while individuals of groups (3) and (4) received only 180 mg.

Proteins can be enzymatically broken down to their amino acids, which in turn are used as substrates in different metabolic pathways. Some amino acids, for instance, can be directly or indirectly (via pyruvate) converted in acetyl-CoA, which is the form acetate is mainly utilized by organisms. Moreover, glucogenic amino acids can be also used to produce glucose in a metabolic pathway called gluconeogenesis (Nelson and Cox 2000). Therefore, individuals of the well nourished groups (1 and 2) should be able to sustain better condition and produce more benzoquinones than individuals of the poorly nourished groups (3 and 4). Moreover, considering that acetate is one of the main precursors of benzoquinones in harvestmen (Rocha et al. 2013), individuals fed with an acetate enriched diet should be able to accelerate the production of benzoquinones because the building blocks of the deterrent compounds are promptly available to them.

After 20 days of diet manipulation, we extracted the secretion produced by individuals of the four experimental groups to analyze the mass and concentration of benzoquinones as described before. Because some individuals died during the experiment, the final number of individuals was slightly different in each group: WNA<sup>-</sup> = 13 males and 11 females; WNA<sup>+</sup> = 11 males and 10 females; PNA<sup>-</sup> = 12 males and 11 females; and PNA<sup>+</sup> = 12 males and 11 females. Despite mortality, the final sample size and the proportion of males and females were rather balanced among all four experimental groups.

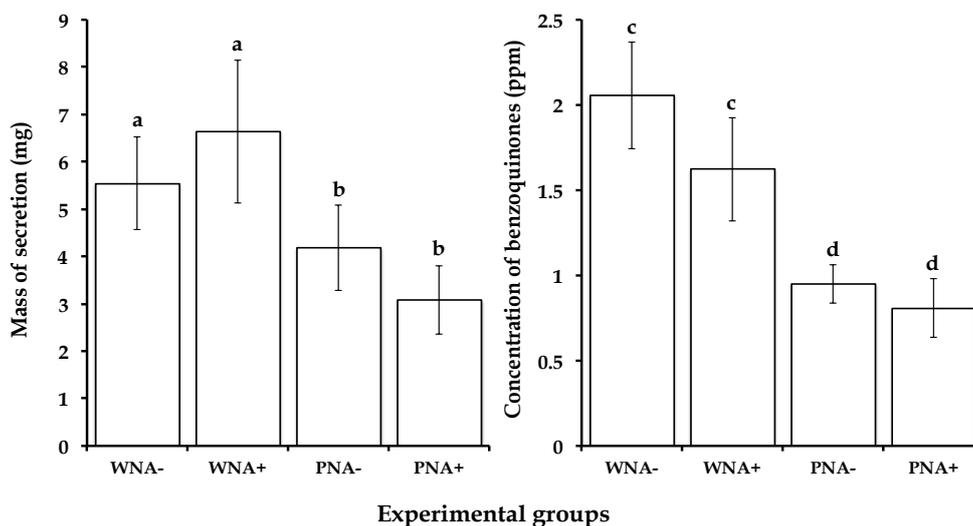
### *Statistical analyses*

First, we compared the initial mass and concentration of benzoquinones produced by individuals of the four experimental groups using an analysis of variance. We also included sex in this analysis in order to explore whether males and females produce defensive secretion with different mass or concentration of benzoquinones. Results of our analyses showed that individuals initially produced the same mass and concentration of benzoquinones regardless of their sex and of the experimental group that they were later allocated to (Table S1). Since the glands of all individuals were completely depleted and

there was no initial difference in the amount of secretion released by individuals of the four groups, we used only the data of the second extraction to compare the experimental groups. Therefore, to test our predictions, we used two analyses of variance in which the response variables were the mass and concentration of benzoquinones in the second extraction, and the predictor variables were sex (with two levels: males and females), food availability (with two levels: WN, PN), diet composition (with two levels: A<sup>+</sup>, A<sup>-</sup>), and their interactions.

## Results

In the first extraction, before the beginning of the experiment, males produced an average ( $\pm$  SD) of  $6.37 \pm 5.21$  mg of secretion, with an average concentration of benzoquinones of  $1.93 \pm 1.37$  ppm. Females produced an average of  $5.7 \pm 5.52$  mg of secretion, with an average concentration of benzoquinones of  $1.68 \pm 1.23$  ppm. As we mentioned before, there was no significant difference between sexes in either mass or concentration of benzoquinones produced (Table S1).



**Fig. 1.** Mean ( $\pm$  SD) values of mass of the secretion (mg) and concentration of benzoquinones (ppm) produced by individuals of the harvestman *Magnispina neptunus* at the end of the experimental period. The four experimental groups were: well nourished without acetate (WNA<sup>-</sup>), well nourished with acetate (WNA<sup>+</sup>), poorly nourished without acetate (PNA<sup>-</sup>), and poorly nourished with acetate (PNA<sup>+</sup>). Since males and females showed quantitatively similar responses (see Table 1), the effect of sex is not shown in the graphics. Different letters indicate significant differences with  $p \leq 0.05$ .

In the second extraction, at the end of the experiment, well nourished individuals produced secretions with greater mass and higher concentration of benzoquinones than poorly fed individuals (Fig. 1) regardless of the sex (Table 1). We detected no significant effect of diet composition in the mass and concentration of secretion produced by individuals of both sexes (Table 1; Fig. 1). Moreover, there was also no significant effect of the interaction between sex, food availability, and diet composition (Table 1).

**Table 1.** Results of the analyses of variance considering the effects of sex, food availability (two levels), and diet composition (two levels) on the mass of secretion and concentration of benzoquinones produced by individuals of the harvestman *Magnispina neptunus* at the end of the experiment (df= degrees of freedom, MS= mean squares, and F = F-statistic). Significant effects are highlighted in bold. See main text for details on the experimental setup.

Source of variation	df	MS	F	p-value
<b>Mass of secretion</b>				
Sex	1	3.70	0.15	0.70
<b>Food availability</b>	<b>1</b>	<b>137.81</b>	<b>5.38</b>	<b>0.02</b>
Diet composition	1	0.09	0.004	0.95
Sex * Food availability	1	0.72	0.03	0.87
Sex * Diet composition	1	27.88	1.09	0.30
Food availability * Diet composition	1	26.95	1.05	0.31
Sex * Food availability * Diet composition	1	0.25	0.01	0.92
Error	83	25.60		
<b>Concentration of benzoquinones</b>				
Sex	1	0.60	0.44	0.51
<b>Food availability</b>	<b>1</b>	<b>21.18</b>	<b>15.65</b>	<b>&lt;0.001</b>
Diet composition	1	1.87	1.38	0.24
Sex * Food availability	1	0.72	0.53	0.47
Sex * Diet composition	1	0.005	0.004	0.95
Food availability * Diet composition	1	0.49	0.36	0.55
Sex * Food availability * Diet composition	1	0.03	0.02	0.88
Error	83	1.35		

## Discussion

The literature on the fitness effects of food availability and diet composition in predatory arthropods has been focused mainly on developing time, female fecundity, and survival rates (e.g., Toft and Wise 1999; Eubanks and Denno 2000; Allard and Yeargan 2005). Some studies with arthropods have also investigated how food availability in particular influences the expression of exaggerated secondary sexual traits, the production of sexual pheromones, and the intensity of immune response in males (e.g., Rantala et al. 2003; Bonduriansky 2007). Although chemical defenses are widespread among arthropods, representing one of the main lines of defense against predators (Eisner et al. 2005), little attention has been devoted to investigate how the production of deterrent compounds respond to condition. Here we designed a laboratory experiment to fill this gap, and our results clearly indicate that well nourished individuals of the harvestman *Magnispina neptunus* produce more defensive secretions than poorly nourished individuals. This finding provides support to our first hypothesis that the production of chemical defenses is condition-dependent.

Recently we showed that non-ovigerous females of the harvestman *Acutisoma longipes* (Gonyleptidae) produce nearly 72% more defensive secretions than ovigerous females, probably as the result of allocation trade-offs between chemical defenses and egg production (Nazareth and Machado 2015). Although the concentration of benzoquinones in the secretions of ovigerous and non-ovigerous females was similar, we showed in a series of bioassays, performed both in the field and in the laboratory, that the greater amount of secretions released by non-ovigerous provide much more effective protection against ants and spiders than the secretion of ovigerous females (Nazareth and Machado 2015). If poorly nourish individuals of *M. neptunus* produce less defensive secretions (both in terms of total mass and concentration), they should also be more exposed to the attacks of predators under natural conditions. Therefore, even periods of dietary restriction as short as 20 days may impose severe fitness consequences to chemically protected arthropods that need to pay the costs of producing their own deterrent compounds. Considering that *M. neptunus* males care for eggs inside natural cavities in roadside banks and during egg attendance their feeding frequency is markedly reduced when compared with non-caring males (Nazareth and Machado 2010), paternal care may expose males not only to foraging costs, but may also compromise their chemical defenses, making them more vulnerable to predation.

The results of our experiment reject our second hypothesis because individuals receiving a diet enriched with benzoquinone precursors (acetate) do not produce more defensive secretions than individuals whose diet did not include promptly available precursors. Unfortunately, our knowledge on harvestman metabolism is very limited, which prevents a thorough interpretation of this finding. We know, however, that acetate is one of the main building blocks in animal metabolism, and it is used both in biosynthetic pathways leading to long chain fatty acids and polyketides (Pankewitz and Hilker 2008). Due to food limitation, which intensifies allocation trade-offs, individuals of the poorly nourished groups probably directed all acetate provided in the diet to the production of vital molecules, including glucose via gluconeogenesis (Nelson and Cox 2000). On the other hand, if the amount of food provided for the individuals in the well nourished groups was more than sufficiently high for basic metabolic activities, the acetate provided in the diet should not substantially improve the production of chemical secretions. In support to this suggestion, food remains were frequently found in the Petri dishes housing well nourished individuals. In the future, a geometric framework (*sensu* Waldbauer and Friedman, 1991) may shed some light on how diet composition may influence the production of chemical defenses in harvestmen and other predatory arthropods.

The fact that males and females showed no initial difference in the amount of secretion released, or in their response to the experimental manipulation of their diets is somehow surprising. Given that females usually enhance their fitness by increasing longevity, while males enhance their fitness by increasing mating rates (Bateman 1948; Trivers 1972), sexual difference in the way resources are allocated to different fitness components should lead to sexual dimorphism in the production of defenses against parasites (Rolff 2002) and perhaps against predators. Although there is no study comparing sex-specific investment in chemical defenses in harvestmen, in other arthropod groups in which this subject has been explored, differences between males and females were frequently reported. For instance, female of several aposematic lepidopteran species are more toxic or emetic than males (Marsh and Rothschild 1974). However, there are some species in which the ability of males to synthesize deterrent compounds is greater than in females (reviewed in Pasteels et al. 1983). Based on the available information, no clear pattern of sexual dimorphism in the production of deterrent compounds seems to emerge from arthropods, but a multi-species comparative approach in groups where chemical defense are widespread could provide a clearer picture and reveal possible evolutionary trends.

In conclusion, the production of alkylated benzoquinones by the harvestman *M. neptunus* is dependent of food availability, suggesting that the production of chemical defenses is a costly fitness-related trait. The main implication of this finding is that food deprivation caused by periods of prey shortage and/or by foraging limitation associated to parental activities could compromise the efficiency of chemical defenses and make individuals more exposed to predators. The role of diet composition in the production of alkylated benzoquinones is still unclear and this subject deserves further investigation.

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## Supplementary Material

**Table S1.** Results of the analyses of variance considering the effects of sex and experimental group on the mass of secretion and concentration of benzoquinones produced by individuals of the harvestman *Magnispina neptunus* before the beginning of the experiment (df= degrees of freedom, MS= mean squares, and F = F-statistic). See main text for details on the experimental setup.

Source of variation	df	MS	F	p-value
<b>Mass of secretion</b>				
Sex	1	0.61	0.12	0.73
Food availability	1	0.65	0.12	0.72
Diet composition	1	5.86	1.13	0.29
Sex * Food availability	1	3.94	0.76	0.39
Sex * Diet composition	1	0.20	0.04	0.84
Food availability * Diet composition	1	1.84	0.35	0.55
Sex * Food availability * Diet composition	1	4.15	0.80	0.37
Error	83	5.2		
<b>Concentration of benzoquinones</b>				
Sex	1	1.45	0.84	0.36
Food availability	1	2.45	1.42	0.24
Diet composition	1	2.02	1.17	0.28
Sex * Food availability	1	0.50	0.29	0.59
Sex * Diet composition	1	2.12	1.23	0.27
Food availability * Diet composition	1	0.29	0.17	0.68
Sex * Food availability * Diet composition	1	0.56	0.33	0.57
Error	83	1.72		

# Capítulo 3

**Does mating effort compromise male investment in chemical defences?  
A comparative study in a clade of Neotropical harvestmen  
(Arachnida: Opiliones)\***

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## **Abstract**

Theory predicts males should invest more resources in mating effort to maximize mating rate, while females should invest more resources in somatic effort to improve survival probability. Thus, females should invest more than males in immune response, which is a key component of somatic effort. Little attention has been paid to other components of the somatic effort that may also increase longevity, such as chemical defences against predators. Considering chemical defences are costly, we tested whether male investment in mating effort promotes a decrease in the production of chemical compounds when compared to females. To test this hypothesis, we used eight harvestman species of the family Gonyleptidae for which we quantified (1) male mating effort as the degree of morphological sexual dimorphism, and (2) sexual dimorphism in the total amount and concentration of benzoquinones released by males and females. Females produced more defensive secretion than males, both in terms of mass and concentration of benzoquinones. However, although negative, we found no significant relationship between the degree of morphological sexual dimorphism and the defensive secretion sexual dimorphism. These findings suggest that the magnitude of potential trade-offs between self-protection and other life-history components may be higher for immune than for chemical defences.

**Key words:** benzoquinones, immune function, life-history trade-off, sexual dimorphism, somatic effort.

## 1. Introduction

Sexual selection imposes different selective pressures on males and females, resulting in marked differences between the sexes in life history strategies and resource allocation [1,2]. Whereas mating rate (i.e., the frequency with which males achieve copulations) is the major determinant for male fitness, female fitness is mainly influenced by food acquisition and longevity, which increase lifetime fecundity [3,4,5]. Therefore, males should invest more resources in mating effort in order to maximize mating rate, and females should invest more resources in somatic effort in order to improve survival probability. Sexual ornaments and weaponry are indeed more frequent in males than in females, suggesting greater male investment in mating effort [6]. Regarding somatic effort, several studies have shown that females are more immunocompetent than males [7]. Given that maintaining and mounting immune responses is costly [8], allocation trade-offs between somatic and mating effort may explain the sexual dimorphism in immunocompetence widely reported in the literature [9].

Although much attention has been dedicated to immune function in recent years, this is only one component of somatic effort in animals. Little or no attention has been paid to other components of the somatic effort that may also increase longevity, such as chemical defences against predators. Like the immune response, chemical defences in arthropods are nonspecific [8], meaning that they are not directed at any natural enemy in particular [10]. Moreover, there is extensive evidence showing that chemical defences increase individual survival by decreasing the chances of that individual being attacked or consumed by a wide range of predators [11, 12]. Finally, both immune response and chemical defences involve high metabolic costs and consequently may impose allocation trade-offs between self-protection and reproduction [13, 14]. In this context, we hypothesize that the same pattern of sexual dimorphism in immune competence should also be found in chemical defences: the greater the degree of male mating effort, the lower the somatic effort in the production of chemical defences, leading to female-biased sexual dimorphism.

Representatives of the order Opiliones, commonly known as harvestmen, are suitable organisms to test the hypothesis presented above for several reasons. First, many species are chemically defended and release a great variety of chemical compounds, including for instance ketones, phenols, and benzoquinones [15]. Second, chemical defences are highly effective, protecting the individuals against both vertebrate and invertebrate predators [16, 17]. Third, there is evidence suggesting that chemical defences in the group are costly [18, see also Chapter 2]. Finally, sexual dimorphism is well marked in many species, with males

exhibiting several conspicuous morphological traits used as weapons in intra-sexual reproductive contests [19].

Although information on female longevity in harvestmen is scarce, we can use the intensity of morphological sexual dimorphism as a proxy of sexual differences in mating effort. According to this rationale, the greater the degree of sexual dimorphism between males and females, with males being larger, the greater should be their investment in mate acquisition [e.g., 20]. Using phylogenetic comparative methods, we tested whether there is a negative correlation between the intensity of morphological sexual dimorphism and the degree of dimorphism in the amount of chemical secretion released by harvestman species of the family Gonyleptidae. The species we selected belong to different subfamilies [21], all release mixtures of benzoquinones as the main defensive compounds [22], and the intensity of morphological sexual dimorphism ranges from nearly indistinguishable males and females until heavily armed males and almost completely unarmed females (Figure S1 in Electronic Supplementary Material).

## 2. Material and methods

### *Data collection*

We collected individuals of eight species belonging to the family Gonyleptidae: *Acantopachylus aculeatus* (Pachylinae), *Acutisoma longipes* (Goniosomatinae), *Bourguyia trochanteralis* (Bourguyiinae), *Cobania picea* (Cobaniinae), *Magnispina neptunus* (Heteropachylinae), *Multumbo terrenus* (Hernandariinae), *Saramacia lucasae* (Manaosbiinae) and *Serracutisoma proximum* (Goniosomatinae). We took individuals of all species to the laboratory, where we induced the release of chemical secretions using the procedure described in Nazareth & Machado (2015) [18]. After extraction, we weighed the amount of secretion released by each individual (to the nearest 0.00001 g) and estimated the total concentration of benzonquinones by gas chromatography (see details in the Electronic Supplementary Material). Average data per species are presented in Table S1 of the Electronic Supplementary Material. To estimate sexual dimorphism in the weight and concentration of defensive secretions released by each species, we calculated a modified version of the Lovich & Gibbons (1992) [23] sexual dimorphism index (SDI), such that positive values mean that males are the sex that produces more defensive secretions.

Given that sexual dimorphism in harvestmen can occur in many different body structures (see Figure S1 in Electronic Supplementary Material), we selected eight traits that were more widespread in the species of our sample and that better characterize their intensity of morphological sexual dimorphism. Most of these traits are used in male-male fights over the possession of breeding territories [24], and thus are likely to be directly related to male mating effort. The traits we measured were: width of the dorsal scute (carapace), length of the external apophysis on the fourth leg coxa, length of the internal apophysis on the fourth leg trochanter, length of the internal apophysis at the tip of the fourth leg femur, length and width of the fourth leg femur, number of apophyses on the fourth leg femur, and total length of the second leg (Figure S2 in Electronic Supplementary Material). To create an overall measure of morphological sexual dimorphism per species, we first calculated the Lovich & Gibbons (1992) [23] index for each trait following the same procedure explained above (values are presented in Table S2 in Electronic Supplementary Material). Then, we estimated an overall sexual dimorphism index by combining all the indexes for each species in a linear model, and used this measure as our proxy of the intensity of morphological sexual dimorphism.

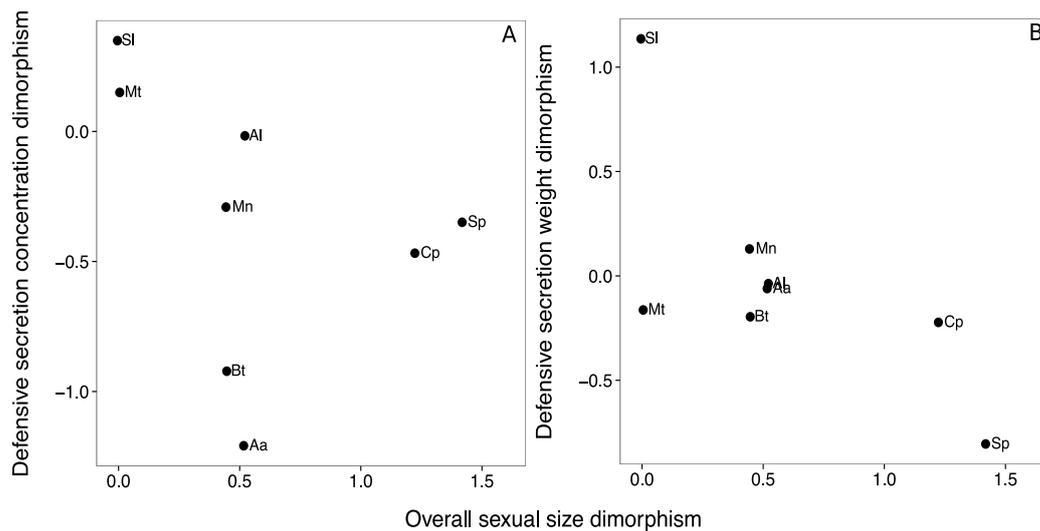
#### *Statistical analyses*

To estimate the effect of our predictor variable (overall morphological sexual dimorphism) on the response variables (defensive secretion dimorphism in concentration and weight) we used Bayesian Markov Chain Monte Carlo (MCMC) phylogenetic linear models implemented in the package *MCMCglmm* [25, 26] in R [27]. We used the phylogenetic tree from Pinto-da-Rocha et al. (2014) [21], which is presented in Figure S3. The pruned tree to our taxa was ultrametric. As an index of phylogenetic signal, we calculated phylogenetic heritability  $H^2$ , which is the ratio of phylogenetic variance to the sum of all other variance components from the model. When the unit of analysis is at the species level, such as in our analysis,  $H^2$  is equivalent to Pagel's  $\lambda$  [28]. We ran the models with and without the phylogenetic tree, and compared models with DIC [29]. Models with the phylogeny had better fit (defensive secretion dimorphism in concentration: model with phylogeny: DIC = -6.212; model without phylogeny: DIC = 19.526; defensive secretion dimorphism in weight: model with phylogeny: DIC = 11.230; model without phylogeny: DIC = 12.230), which suggests that phylogenetic relatedness explains residual variance; therefore, the main models reported in the results account for phylogeny. We used the inverse Gamma prior ( $V = 0.002$ , and  $nu = 1$ ) for the random effect. Models were run for 3,000,000 iterations with a thinning

interval of 1,000 after a burn-in of 2,000,000 iterations. We used these settings to obtain posterior distributions for all parameters from 1,000 samples. We considered chain mixing by examining autocorrelation among posterior samples ( $< 0.1$  for all parameters) [25]. We checked model convergence by examining the Gelman-Rubin statistic among three chains (potential scale reduction factor  $< 1.1$  for all fixed and random parameters) [30]. We report point estimates as posterior means, and considered fixed effects statistically significant if the 95% credible interval (CI) did not overlap zero.

### 3. Results

Both the concentration and the weight of the secretion were extremely variables among species and sexes (Figure 1). On average females produced defensive secretions with significantly greater mass than males (intercept = 10.969, 95% CI: 3.965 to 17.914;  $\beta_{(\text{Male})} = -1.872$ , 95% CI: -2.947 to -0.777), and significantly higher concentration than males (intercept = 27.104, 95% CI: 4.528 to 52.491;  $\beta_{(\text{Male})} = -9.395$ , 95% CI: -12.909 to -6.148; Table S1). However, there was little evidence of a negative effect of average morphological sexual size dimorphism on the defensive secretion concentration dimorphism, as the 95% CI overlapped zero (intercept = -0.220, 95% CI: -1.136 to 0.697;  $\beta_{(\text{SSD})} = -0.114$ , 95% CI: -1.114 to 0.857; Figure 1A). Phylogenetic heritability in the model was strong, but note the wide 95% CI ( $H^2 = 0.995$ , 95% CI: 0.007 to 0.999). There was a moderate negative, yet non-significant, effect of sexual size dimorphism on the defensive secretion weight dimorphism (intercept = 0.373, 95% CI: -0.345 to 1.085;  $\beta_{(\text{SSD})} = -0.711$ , 95% CI: -1.640 to 0.132; Figure 1B). In this case, phylogenetic heritability in the model was weak, but again with a wide 95% CI ( $H^2 = 0.007$ , 95% CI: 0.0003 to 0.836).



**Figure 1:** Relationship between the average sexual morphological dimorphism and the sexual dimorphism in the (A) concentration and (B) mass of defensive secretion released by eight harvestman species of the family Gonyleptidae. *Acantopachylus aculeatus* (Aa), *Acutisoma longipes* (Al), *Bourguyia trochanteralis* (Bt), *Cobania picea* (Cp), *Magnispina neptunus* (Mn), *Multumbo terrenus* (Mt), *Saramacia lucasae* (Sl), and *Serracutisoma proximum* (Sp).

#### 4. Discussion

Here we tested whether male investment in male mating effort imposes a reduction in the investment in chemical defences as a result of allocation trade-offs between reproduction and self-protection. As should be expected, harvestman females produced more defensive secretion than males, both in terms of mass and concentration of benzoquinones. However, although negative, we found no significant relationship between the degree of morphological sexual dimorphism and the defensive secretion sexual dimorphism. Therefore, we found weak support for the hypothesis of allocation trade-offs between male mating effort and self-protection in terms of production of chemical secretions in gonyleptid harvestmen.

Despite the fact that immune responses and production of chemical secretions involve high metabolic costs, there is a fundamental difference between these two types of defences. While both impose costs of having and maintaining the biochemical machinery necessary for the defence against natural enemies, only the immune system seems to require resources for mounting an effective response after an attack [31]. In fact, there is extensive evidence showing that mounting an immune response after an experimental challenge is costly, imposing life-history trade-offs [reviewed in 31]. In the case of chemical secretions, the costs of activating the defensive machinery is likely to be negligible when compared to

mounting an immune response [11]. Moreover, if predation pressure is low, the costs of replenishing chemical secretions after discharge are expected to be low, reducing the costs of maintaining the chemical defences [32]. Thus, the magnitude of potential trade-offs between self-protection and other life-history components may be higher for immune than for chemical defences. It could explain why higher male investment in mating effort does not impose a marked reduction in the production of chemical secretions in harvestmen.

Regardless of the degree of morphological sexual dimorphism, harvestman males produced less chemical secretions than females. This finding indicates that sexual dimorphism in the production of chemical defences follows the same general pattern reported for the immune system in invertebrates, in which females usually exhibit more pronounced responses than males [reviewed in 7]. Females of several aposematic lepidopteran species are more toxic or emetic than males, suggesting that they also invest more in chemical defences than males [33]. Since the amount of chemical secretions in harvestmen is positively related to their effectiveness against predators [18], males are expected to be more vulnerable to predation than females. For one species studied here, *Serracutisoma proximum*, females produce 80.5% more secretion than males, and long-term mark-recapture studies in the field allow precise estimation of individual survival [see 34, 35]. Contrary to what should be expected, monthly female survival probability (mean  $\pm$  SE =  $0.857 \pm 0.022$ ) is not higher than males ( $0.812 \pm 0.028$ ). However, more information on other species are necessary to investigate whether there is a general pattern of sex-biased mortality in chemically defended arthropods.

We are aware that our sample size here is rather limited, and ideally more species should be included in the comparative analysis. As a way of circumventing this problem, we selected species exhibiting great interspecific variation in morphological sexual dimorphism in order to spread the data along the axis of the predictor variable. Even using this procedure, the final dataset still has some gaps and there is no species with morphological sexual dimorphism between 0.1 and 0.4, and between 0.6 and 1.2. Despite these problems, we detected a general negative, though non-significant, relationship between the morphological sexual dimorphism and the chemical secretion sexual dimorphism. To increase the statistical power of the comparative analysis, our plan is: (1) to expand the number of benzoquinone-producing species included in the dataset, (2) to increase the number of gonyleptidae clades represented in the dataset, and (3) to fill the gaps of morphological sexual dimorphism. Based on these criteria, the potential targets are *Discocyrtus invalidus*, *Hypophyllonemus longipes*, one species of the genus *Eusarcus*, and one

representative of the subfamily Cranainae because these taxa are all included in the recent phylogeny of Gonyleptidae [21]. The inclusion of these species will allow more robust inference on the existence of allocation trade-offs between male mating effort and self-protection in terms of production of chemical secretions in harvestmen.

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## Electronic Supplementary Material

### *Concentration of benzoquinones*

The defensive secretion from each individual was obtained by holding its legs and pressing the dorsal scute (carapace) with a small piece of decontaminated cotton wool ball held by tweezers. The procedure was repeated until the gland was completely emptied. Each piece of cotton, which was previously weighed (dry weight), was weighed again, thus we obtained the mass of the secretion (difference between wet and dry cotton wool weight). For the quantitative determination of quinone, the cotton wool ball was rinsed from 2 to 4 times with  $\text{CH}_2\text{Cl}_2$ , in such a way that each wash added 500  $\mu\text{L}$  to the cotton depending on the species that was analyzed. Then we added, in each sample, benzophenone as an internal standard, in a known concentration (which varied among species). Samples were analyzed by gas chromatography coupled to flame ionization detection.

The machine we used was a Shimadzu GC-FID 2014 gas chromatograph coupled with an AOC20i autosampler fitted with a RTX-5 capillary column (30.0 m  $\times$  0.32 mm, 5% phenyl, 95% dimethylpolysiloxane). The oven temperature program was as follows: 40  $^\circ\text{C}$  (2 min), and then increased first to 200  $^\circ\text{C}$  at 5  $^\circ\text{C}/\text{min}$  (1 min). Nitrogen (99.999%, White Martins) was used as the carrier gas at a linear velocity, column flow and purge of 18.7  $\text{cm s}^{-1}$ , 1  $\text{mL min}^{-1}$  and 3  $\text{mL min}^{-1}$ , respectively. Injections of 1  $\mu\text{L}$  were carried out in a splitless mode, during 1 min, at 220 $^\circ\text{C}$  and 32.5 kPa. Dichloromethane (absolv grade, Tedia) was used as injection solvent and the syringe cleaning solvent between injections. Temperature, air (20%  $\text{O}_2$  in  $\text{N}_2$ , Linde) and hydrogen (99.999%, White Martins) flows of detector were set at 250  $^\circ\text{C}$ , 400 and 40  $\text{ml min}^{-1}$ , respectively.

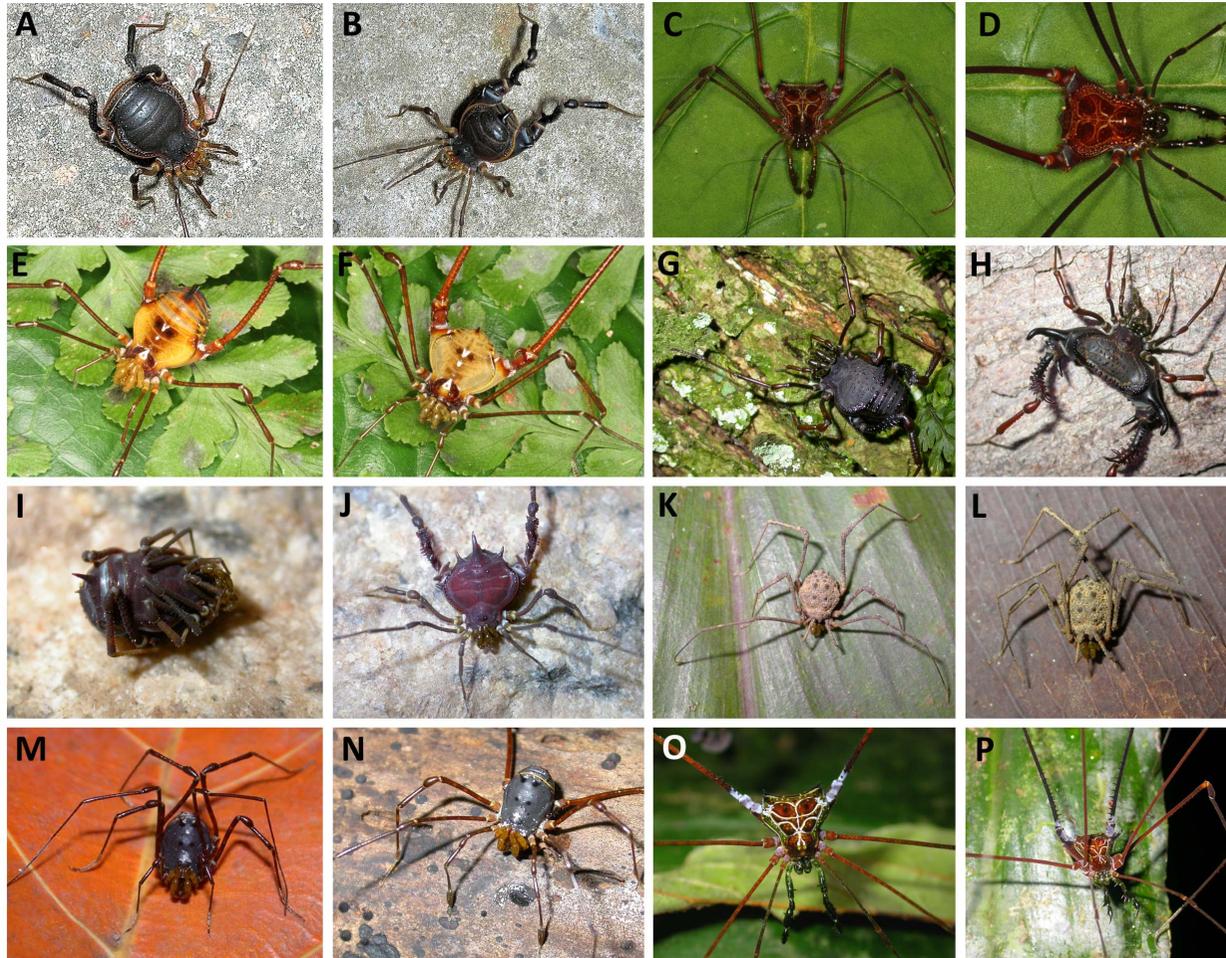
We calculated the relative amount of the benzoquinones contained in each sample by the ratio between the area and mass of internal standard and the area of identified benzoquinones in each sample. Total benzoquinones per sample was the sum of the net quantities of the benzoquinones present in the mixture. We quantified benzoquinones from the linear regression equation, for each species, of a calibration curve constructed for 1,4-benzoquinone, so that all amounts are expressed as 1,4- benzoquinone equivalents.

**Table S1:** Harvestman species included in the analysis, with the identity of benzoquinones present in their defensive secretions, the total weight and concentration of benzoquinones released by males and females, and the location where each species was collected. Abbreviations:  $\bar{x}$ =Average, SD = Standard deviation, and n = Number of individuals.

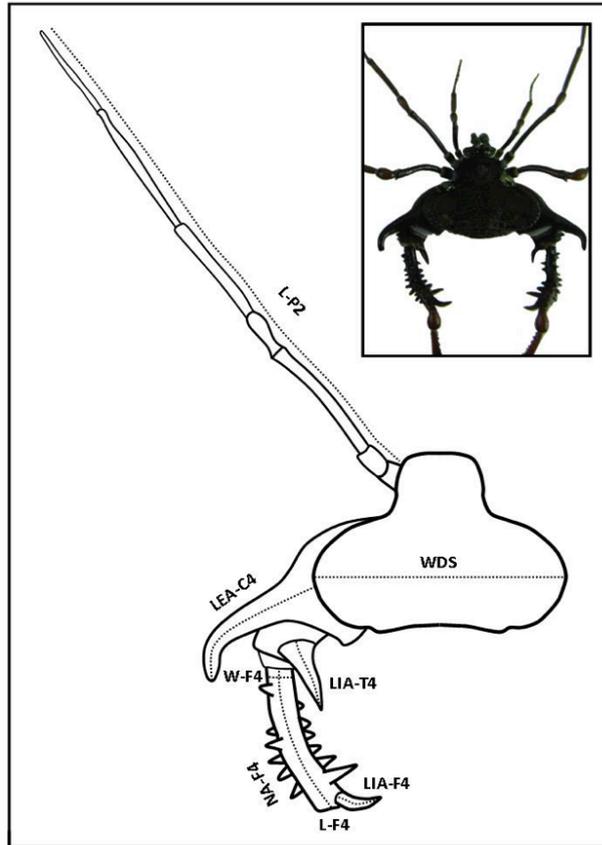
Species	Molecules	Weight of total benzoquinones (mg)			Concentration of total benzoquinones (ppm)			Collection locality
		$\bar{x}$	SD	n	$\bar{x}$	SD	n	
<i>Acanthopachylus aculeatus</i> (♀)	2,3-dimethyl-1,4-benzoquinone	6.53	2.06	35	23.87	19.63	35	Montevideo, Uruguay
<i>Acanthopachylus aculeatus</i> (♂)	2,5-dimethyl-1,4-benzoquinone							
<i>Acanthopachylus aculeatus</i> (♂)	2,3,5-trimethyl-1,4-benzoquinone	6.16	2.14	47	10.81	10.05	48	
<i>Acutisoma longipes</i> (♀)	2,3-dimethyl-1,4-benzoquinone	19.02	11.80	58	20.29	14.43	86	Atibaia, São Paulo, Brazil
<i>Acutisoma longipes</i> (♂)	2-ethyl-3-methyl-1,4-benzoquinone	18.34	7.49	32	19.95	11.79	33	
<i>Bourguyia trochanteralis</i> (♀)	2,3-dimethyl-1,4-benzoquinone 2-ethyl-3-methyl-1,4-benzoquinone 2,3,5-trimethyl-1,4-benzoquinone	8.69	2.96	33	31.84	17.49	33	Ilha do Cardoso, São Paulo, Brazil
<i>Bourguyia trochanteralis</i> (♂)	2-ethyl-3,5-dimethyl-1,4-benzoquinone or 2-ethyl-3,6-dimethyl-1,4-benzoquinone	7.27	3.68	17	16.57	10.79	17	
<i>Cobania picea</i> (♀)	2,3-dimethyl-1,4-benzoquinone	21.04	9.66	60	108.44	50.81	60	Itamonte, Minas Gerais, Brazil
<i>Cobania picea</i> (♂)	2-ethyl-3-methyl-1,4-benzoquinone	17.21	7.93	43	73.97	33.47	43	
<i>Magnispina neptunus</i> (♀)	2-methyl-1,4-benzoquinone	3.68	1.89	58	1.79	1.50	58	Arraial D'Ajuda, Bahia, Brazil
<i>Magnispina neptunus</i> (♂)	2-ethyl-1,4-benzoquinone	4.15	3.63	66	1.38	1.13	66	
<i>Multumbo terrenus</i> (♀)	2,5-dimethyl-1,4-benzoquinone 2-ethyl-3-methyl-1,4-benzoquinone 2-ethyl-5-methyl-1,4-benzoquinone 2,3,5-trimethyl-1,4-benzoquinone	4.67	2.62	36	10.52	8.30	36	Teresópolis, Rio de Janeiro, Brazil
<i>Multumbo terrenus</i> (♂)	2-ethyl-3,5-dimethyl-1,4-benzoquinone or 2-ethyl-3,6-dimethyl-1,4-benzoquinone	4.02	1.63	29	12.09	7.40	29	
<i>Saramacia lucasae</i> (♀)	2,3-dimethyl-1,4-benzoquinone	5.77	3.66	10	0.0017	0.001	10	Manaus, Amazonas, Brazil
<i>Saramacia lucasae</i> (♂)	2-ethyl-3-methyl-1,4-benzoquinone	6.76	2.94	10	0.0022	0.002	10	
<i>Serracutisoma proximum</i> (♀)	2,3-dimethyl-1,4-benzoquinone	26.65	7.63	21	15.87	3.69	21	Ribeirão Grande, São Paulo, Brazil
<i>Serracutisoma proximum</i> (♂)	2-ethyl-3-methyl-1,4-benzoquinone	14.76	5.18	24	11.76	5.14	24	

**Table S2:** Average values of the morphological traits (in mm) used to estimate sexual dimorphism in the selected species. LIA-T4 = Length of the internal apophysis on fourth leg trochanter, LEA-C4 = Length of the external apophysis on the fourth leg coxa, LIA-F4 = Length of the internal apophysis at the tip of fourth leg femur, L-F4 = Length of the fourth leg femur, L-L2 = Length of the second leg, NA-F4 = Number of apophyses on the fourth leg femur, WDS = Width of the dorsal scute (carapace), and W-F4 = Width of the fourth leg femur. Abbreviations:  $\bar{X}$  = Average, SD = Standard deviation, and n = Number of individuals.

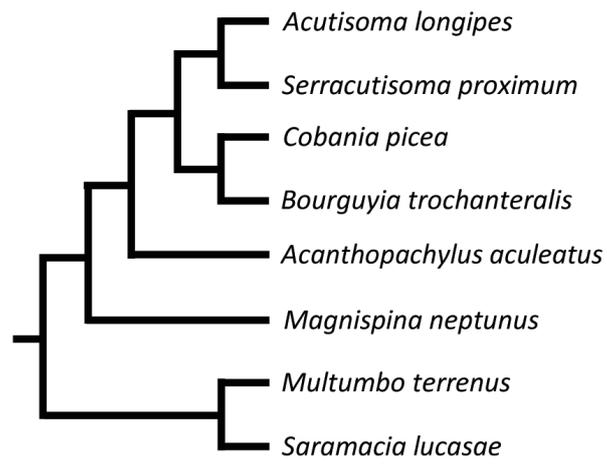
Species	LIA-T4			LEA-C4			LIA-F4			L-F4			L-L2			NA-F4			WDS			W-F4		
	$\bar{X}$	SD	n	$\bar{X}$	SD	n	$\bar{X}$	SD	n	$\bar{X}$	SD	n	$\bar{X}$	SD	n	$\bar{X}$	SD	n	$\bar{X}$	SD	n	$\bar{X}$	SD	n
<i>A. aculeatus</i> (♀)	0.19	0.19	15	0.66	0.14	46	0.57	0.07	21	5.28	0.58	59	15.99	1.30	59	6	0	13	6.49	0.53	40	0.76	0.06	21
<i>A. aculeatus</i> (♂)	-	-	-	2.06	0.50	42	0.82	0.11	15	5.53	0.66	40	17.81	1.63	40	7	0	7	6.74	0.86	49	1.28	0.17	15
<i>A. longipes</i> (♀)	-	-	-	1.47	0.19	58	0.21	0.02	8	22.42	1.36	85	73.55	10.1	144	-	-	-	9.33	0.69	86	0.74	0.05	8
<i>A. longipes</i> (♂)	1.59	1.59	15	3.07	0.52	78	0.58	0.13	15	24.61	1.73	78	80.94	9.06	91	-	-	-	9.49	1.09	33	0.84	0.09	15
<i>B. trochanteralis</i> (♀)	0.29	0.07	16	0.99	0.14	39	0.28	0.07	15	12.93	2.28	39	34.73	1.94	33	2	0	9	7.21	0.72	33	0.47	0.05	16
<i>B. trochanteralis</i> (♂)	0.56	0.13	13	1.72	0.58	37	0.19	0.04	8	21.92	4.24	37	49.53	5.00	17	4	0	7	8.51	1.70	17	0.54	0.06	13
<i>C. picea</i> (♀)	0.59	0.07	15	1.37	0.19	90	0.91	0.12	15	6.93	0.56	89	25.09	3.22	59	5.53	0.52	15	10.32	0.67	60	1.07	0.14	15
<i>C. picea</i> (♂)	1.82	0.73	15	5.55	2.42	101	1.71	0.54	15	7.61	1.39	101	25.84	5.08	42	22.7	0.52	6	12.05	2.08	43	1.51	0.29	15
<i>M. neptunus</i> (♀)	0.30	0.05	17	0.53	0.11	64	0.51	0.06	18	4.37	0.47	64	14.60	0.97	54	8	0	5	6.49	0.34	58	0.40	0.05	18
<i>M. neptunus</i> (♂)	0.65	0.12	23	0.91	0.15	115	0.37	0.06	23	4.59	0.48	112	15.78	1.35	65	13	0	8	7.27	0.59	66	0.89	0.07	23
<i>M. terrenus</i> (♀)	-	-	-	-	-	-	-	-	-	7.54	0.34	36	20.79	0.64	36	-	-	-	5.41	0.38	35	0.46	0.02	36
<i>M. terrenus</i> (♂)	-	-	-	-	-	-	-	-	-	7.51	0.37	29	20.99	0.73	29	-	-	-	5.27	0.31	28	0.46	0.02	29
<i>S. lucasae</i> (♀)	0.22	0.05	10	0.51	0.07	10	0.21	0.06	10	7.12	0.28	10	26.30	0.70	10	-	-	-	4.88	0.16	10	0.42	0.06	10
<i>S. lucasae</i> (♂)	0.22	0.05	10	0.56	0.05	10	0.180	0.05	10	7.26	0.17	10	26.99	0.76	10	-	-	-	4.61	0.14	10	0.39	0.04	10
<i>S. proximum</i> (♀)	0.23	0.04	18	0.78	0.05	21	0.21	0.02	10	22.48	0.87	443	88.79	3.48	382	5	0	8	8.58	0.59	21	0.84	0.04	18
<i>S. proximum</i> (♂)	0.41	0.09	17	3.59	0.41	62	0.26	0.04	10	25.77	1.82	365	127.7	17.5	389	35	0	10	8.59	0.53	24	0.88	0.11	17



**Figure S1:** Photos of the species included in our dataset: (A-B) *Acantopachylus aculeatus* (photos: C. Toscano-Gadea), (C-D) *Acutisoma longipes* (photos: R. Pinto-da-Rocha), (E-F) *Bourguyia trochanteralis* (photos: R. Pinto-da-Rocha), (G-H) *Cobania picea* (photos: B.A. Buzatto), (I-J) *Magnispina neptunus*, (K-L) *Multumbo terrenus*, (M-N) *Saramacia lucasae*, and (O-P) *Serracutisoma proximum* (photos: B.A. Buzatto). For all species, females are in the left and males in the right.



**Figure S2:** Scheme of a *Cobania picea* male showing the morphological traits that were measured to estimate sexual dimorphism. The inset shows a photo of a male of this species. LIA-T4 = Length of the internal apophysis on fourth leg trochanter, LEA-C4 = Length of the external apophysis on the fourth leg coxa, LIA-F4 = Length of the internal apophysis at the tip of fourth leg femur, L-F4 = Length of the fourth leg femur, L-L2 = Length of the second leg, NA-F4 = Number of apophyses on the fourth leg femur, WDS = Width of the dorsal scute (carapace), and W-F4 = Width of the fourth leg femur.



**Figure S3:** Phylogenetic relationship of the harvestman species included in the dataset. The topology is based on Pinto-da-Rocha et al. (2014).

## Conclusão geral

Esta tese foi pensada para explorar uma lacuna importante em nosso conhecimento sobre ecologia química: quais são os custos de produzir compostos químicos defensivos em artrópodes? Para isso, selecionamos várias espécies de opiliões como modelo de estudo e conduzimos tanto estudos experimentais quanto análises comparativas. A seguir, apresentamos as principais conclusões alcançadas em cada um dos capítulos que fizeram parte da tese.

No capítulo 1, investigamos como a produção de ovos pode comprometer a produção de defesas químicas compostas por benzoquinonas e quais são as consequências da redução da quantidade de secreção liberada sobre a capacidade de defesa das fêmeas de *Acutisoma longipes* (Gonyleptidae). Nossos resultados apontam que a quantidade de secreção produzida por fêmeas ovígeras é quase 50% inferior à das fêmeas não-ovígeras, indicando que existe uma demanda conflitante (*trade-off*) entre produção de ovos e de defesas químicas. Se a produção de defesas químicas não fosse custosa, não esperaríamos que a produção de benzoquinonas fosse ser diminuída durante a produção de ovos. Adicionalmente, demonstramos experimentalmente que a secreção liberada por fêmeas ovígeras é menos eficiente em deter inimigos naturais, tais como formigas e aranhas. Portanto, a produção de ovos parece ser um momento crítico na vida das fêmeas, representando talvez um dos maiores custos da reprodução em opiliões.

No capítulo 2, investigamos como a quantidade e a qualidade da dieta influenciam a produção de defesas químicas compostas por benzoquinonas em *Magnispina neptunus* (Gonyleptidae). Demonstramos que a produção de defesas químicas é dependente da condição, pois indivíduos (tanto machos quanto fêmeas) bem alimentados produziram mais secreção do que indivíduos mal alimentados. Entretanto, indivíduos que receberam um aporte extra de acetato, molécula precursora das benzoquinonas em opiliões, não incrementaram nem a quantidade total nem a concentração da secreção defensiva. Considerando que machos de *M. neptunus* reduzem drasticamente a frequência de forrageio durante o período em que estão cuidando dos ovos, a restrição alimentar imposta pelo cuidado paternal e a consequente redução na quantidade de defesas químicas produzidas podem fazer com que os indivíduos cuidadores estejam mais vulneráveis à predação.

No capítulo 3, investigamos se possíveis demandas conflitantes entre o esforço de acasalamento e o esforço somático geram um padrão macro-evolutivo segundo o qual o incremento no dimorfismo sexual na produção de defesas químicas está inversamente correlacionado com o dimorfismo sexual morfológico. Usando uma abordagem comparativa com oito espécies da família Gonyleptidae, mostramos que fêmeas produzem mais defesas químicas que machos, tanto em massa quanto em concentração de benzoquinonas. Entretanto, não houve uma relação negativa entre o dimorfismo sexual na produção de defesas químicas e o dimorfismo sexual morfológico, que foi nossa variável operacional (*proxy*) para o esforço de acasalamento dos machos. Esses resultados indicam que machos e fêmeas diferem em relação ao investimento em defesas, provavelmente porque as demandas para diferentes componentes de aptidão não são as mesmas em cada sexo. Adicionalmente, um maior investimento masculino em armamentos não parece comprometer severamente a produção de defesas químicas, ao contrário do que já foi registrado para defesas imunológicas em vertebrados e invertebrados. Aparentemente, os custos de manutenção e ativação do sistema imune em machos são superiores aos custos de produção e liberação de defesas químicas.

Coletivamente, os resultados obtidos nesta tese apontam que a produção de defesas químicas em opiliões é custosa e que está sujeita a demandas conflitantes com outros componentes de aptidão. Mais especificamente, o investimento em reprodução pelas fêmeas parece corresponder a um dreno importante de energia e recursos, que onera o investimento em defesas químicas. Em algumas espécies em que os machos cuidam dos ovos, o investimento em cuidado parental impede os indivíduos cuidadores de forragear e a restrição alimentar também pode ter efeitos negativos na produção de defesas químicas. Por fim, o maior investimento em acasalamento feito pelos machos implica no desenvolvimento e manutenção de estruturas supostamente custosas, tais como espinhos, tubérculos e a musculatura associada ao quarto par de pernas, comumente usado em brigas entre machos. O alto investimento masculino na busca por parceiras sexuais parece comprometer o investimento somático, tanto em imunidade quanto na produção de defesas químicas.

Ao mesmo tempo que muitas questões foram respondidas, várias outras questões ficaram em aberto e surgiram muitas oportunidades de estudos futuros. A partir dos resultados obtidos no capítulo 1, prevemos que o período de maior vulnerabilidade das fêmeas de *A. longipes* ocorre durante a produção de ovos. Tal previsão pode ser testada por meio de estudos de marcação-recaptura em campo.

Estudos prévios com diferentes espécies de opiliões já usaram procedimentos de marcação-recaptura para estimar a sobrevivência dos indivíduos e, portanto, o grupo oferece as condições ideais para avançar nosso conhecimento sobre as consequências das demandas conflitantes entre produção de ovos e produção de defesas químicas. Da mesma forma, seria possível usar estudos de marcação-recaptura para investigar se a mortalidade de machos cuidadores de *M. neptunus* é maior durante o período de cuidado parental. Finalmente, os resultados apresentados no capítulo 3 devem ser vistos como preliminares, pois mais espécies precisam ser incluídas na análise comparativa a fim de obtermos conclusões mais robustas. Já temos espécies-alvo bem estabelecidas e nosso plano é adicionar de quatro a seis espécies ao bando de dados antes da submissão do trabalho para a publicação. De qualquer forma, em seu estado atual, nosso trabalho aponta para um claro dimorfismo sexual na produção de defesas químicas que suscita duas questões básicas que deverão ser exploradas em mais detalhes no futuro: (1) Qual é a base mecânica (causa proximal) para o menor investimento dos machos em defesas químicas? (2) Quais são as consequências em termos de exposição à predação do menor investimento masculino em defesas químicas? Claramente, a investigação dos custos da produção de defesas químicas em artrópodes promete ser uma jornada tão estimulante quanto aquela trilhada por vários pesquisadores que investigaram seus benefícios. Esperamos, portanto, que os trabalhos apresentados aqui instiguem ecólogos químicos a investigar não apenas os benefícios, mas também custos associados às defesas químicas em animais.