

Chemical Defense as a Condition-Dependent Trait in Harvestmen

Taís M. Nazareth¹ · Daniela B. Sudatti² · Glauco Machado¹

Received: 11 November 2015 / Revised: 17 July 2016 / Accepted: 28 July 2016
© Springer Science+Business Media New York 2016

Abstract The expression of costly traits often depends on the amount of food available to the individuals. Chemical defenses are costly, thus their production should be condition-dependent. Here, we tested the hypothesis that an increase in food availability and an acetate-supplemented diet will increase the production of chemical defenses by the harvestman *Magnispina neptunus*, which releases alkylated benzoquinones biosynthesized using acetate as a precursor. We manipulated the diet of the individuals and created four experimental groups: well-fed with acetate, well-fed without acetate, poorly-fed with acetate, and poorly-fed without acetate. Well-fed individuals produced secretions with higher mass and concentration of benzoquinones than poorly-fed individuals, but we detected no significant effect of the acetate supplement. Thus, the production of benzoquinones is condition-dependent, and even short periods of dietary restriction may make individuals more vulnerable to predators, imposing fitness consequences to chemically-protected arthropods that biosynthesize their own defensive compounds.

Keywords Benzoquinone · Costs · Dietary restriction · Food availability · Parental care · Arachnida · Opiliones

Electronic supplementary material The online version of this article (doi:10.1007/s10886-016-0749-0) contains supplementary material, which is available to authorized users.

✉ Glauco Machado
glaucom@ib.usp.br

¹ LAGE do Departamento de Ecologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, SP 05508–090, Brazil

² Departamento de Biologia Marinha, Instituto de Biologia, Universidade Federal Fluminense, PO Box 100.644, Niterói, Rio de Janeiro 24.001–970, Brazil

Introduction

For animals, “condition” is defined as the amount of food available to an individual, and the efficiency with which food is converted into metabolically useful forms (Rowe and Houle 1996). Many studies have demonstrated that the expression of costly traits is highly dependent on the amount of food available to the individuals (Bonduriansky 2007). Chemical defenses are costly, thus the production of defensive compounds should be condition-dependent. However, few studies have investigated whether the production of chemical defenses is dependent on food availability in species that produce their own defensive compounds (Kogel et al. 2012; Palottini et al. 2014 and references therein).

Chemical defenses are highly effective in arthropods, but they must be replenished frequently (Whitman et al. 1994). Only individuals in good condition should be able to afford the costs of constant production. A suitable group of arthropods to test this hypothesis are arachnids of the order Opiliones, commonly known as harvestmen. Harvestmen have a pair of large exocrine glands located at the anterior margins of the carapace that release defensive compounds. Among species of the superfamily Gonyleptoidea, many species release alkylated benzoquinones, which are highly effective repellents of ants, spiders, frogs, and lizards (Gnaspini and Hara 2007).

Here, we tested how food availability and an acetate-supplemented diet affect the production of chemical defenses by the harvestman *Magnispina neptunus* (Gonyleptidae), which releases a secretion composed of 2-methyl-1,4-benzoquinone and 2-ethyl-1,4-benzoquinone (Rocha et al. 2013; Fig. S1). We hypothesize that well-fed individuals will sustain better condition and produce more benzoquinones than poorly-fed individuals. Moreover, considering that acetate is one of the main precursors of benzoquinones in *M. neptunus*

(Rocha et al. 2013), individuals fed with an acetate-supplemented diet should be able to accelerate the production of secretion due to the availability of the building blocks of the defensive compounds.

Methods and Materials

Collection of Individuals In August 2014, we collected 60 males and 60 females of *M. neptunus* in Arraial d'Ajuda, Bahia, Brazil (SISBIO permit #38,607-1)—the same place where we collected individuals for the study on chemical characterization and biosynthesis of the chemical secretion in the species (Rocha et al. 2013). After collection, we transferred all individuals to the laboratory, where we kept them individually in small Petri dishes containing a piece of wet cotton to maintain humidity.

Quantification of Benzoquinones Before the experiment, we emptied the glands of all individuals to estimate the total mass (mg) and concentration (μg of benzoquinones/mg of secretion) of defensive secretions released by them. First, we seized each individual by hand and induced the emission of defensive secretion by pressing a piece of cotton against the ozopores three times to ensure complete gland depletion. Then, we washed the cotton soaked with secretion in 500 μl of CH_2Cl_2 twice, and spiked this solution with benzophenone (Sigma-Aldrich®) as internal standard (IS). Finally, we injected the resulting solution into a Shimadzu 2014 gas chromatograph coupled with an AOC20i autosampler fitted with a RTX-5 capillary column to perform the quantification of benzoquinones (see equipment settings in Supplementary Material).

We used $^1\text{H-NMR}$ spectrometry to confirm that the two benzoquinones obtained here for *M. neptunus* were the same previously described by Rocha et al. (2013). The methods and results are presented in the Supplementary Material (Fig. S1). For the quantification of 2-methyl-1,4-benzoquinone and 2-ethyl-1,4-benzoquinone, we used the technique of internal normalization adding a 12.1 μg of benzophenone in all samples. We used 1,4-benzoquinone (Sigma-Aldrich®) as external standard (ES), assuming that all benzoquinone components would respond in the detector with the same sensibility.

We prepared standard stock solutions of benzophenone and 1,4-benzoquinone at 1.21 mg/ml and 1.12 mg/ml, respectively, and used them for analytical curve and spiking solutions. We performed the evaluation of the calibration curves' linearity based on injections of seven different solutions, and obtained the calibration curves by plotting the injected concentration of ES/IS against the peak area of ES/IS. Then, we calculated the quantity of each benzoquinone per sample from the obtained linear regression ($R^2 = 0.999$), and expressed as 1,4-benzoquinone

equivalents (Nazareth and Machado 2015). Finally, we calculated the total mass and concentration of benzoquinones per sample summing the mass and concentration of the two benzoquinones present in the secretions.

Experimental Setup We randomly divided the individuals into four experimental groups with 15 males and 15 females each: (1) well-fed without acetate supplement (WFA^-): individuals fed every 2 d with 60 mg of whey protein isolate (Iso Whey Protein®, protein concentration = 97 %) diluted in a drop of water; (2) well-fed with acetate supplement (WFA^+): individuals fed every 2 d with 40 mg of whey protein isolate and 20 mg of pure sodium acetate (Sigma-Aldrich®) diluted in a drop of water; (3) poorly-fed without acetate supplement (PFA^-): individuals fed once every 6 d with the same diet as group WFA^- ; and (4) poorly-fed with acetate supplement (PFA^+): individuals fed once every 6 d with the same diet as group WFA^+ . After a 20 d-period of diet manipulation, individuals from the two well-fed groups had received a total of 600 mg of food, whereas individuals from the two poorly-fed groups had received 180 mg. Although individuals of *M. neptunus* naturally feed on dead and live arthropods, we used only protein and the acetate supplement in the experiment to ensure that the diet of all individuals would be identical and standardized. Proteins can be converted into glucose via gluconeogenesis, which usually is associated with fasting periods.

At the end of the experiment, we extracted the secretion produced by individuals of all groups to quantify the mass and concentration of total benzoquinones using the same methods described before (Fig. S2). Because some individuals died during the experiment, the final number of individuals (males; females) in each group was: $\text{WFA}^- = 13; 11$; $\text{WFA}^+ = 11; 10$; $\text{PFA}^- = 12; 11$; and $\text{PFA}^+ = 12; 11$.

Statistical Analyses We compared the mass and concentration of total benzoquinones produced by individuals from the four groups before and after the experiment using repeated measures analyses of variance, in which time (before and after), sex (males and females), food availability (well-fed and poorly-fed), and acetate supplement (present and absent) were the independent variables. In Tables S1-S2 and Figs. S4-S5 of the Supplementary Material, we present the results for each benzoquinone individually.

Results

Before the experiment, there was no significant difference between sexes and experimental groups either in the mass or concentration of total benzoquinones (Fig. 1, Table 1). At the end of the experiment, well-fed individuals produced secretions with higher mass and concentration of benzoquinones

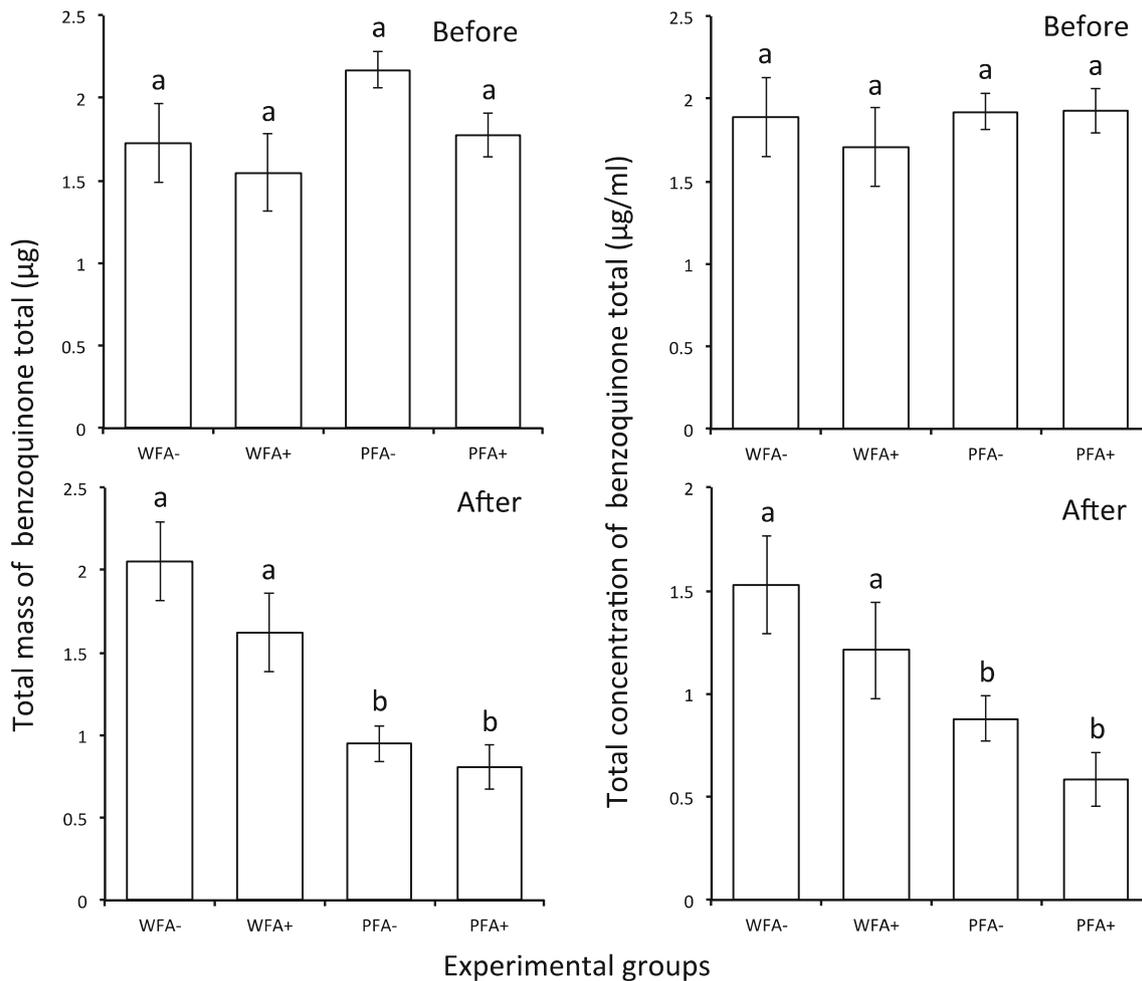


Fig. 1 Mean (\pm SD) mass and concentration of total benzoquinones produced by the harvestman *Magnispina neptunus*. The four experimental groups are: well-fed without acetate supplement (WFA⁻), well-fed with acetate supplement (WFA⁺), poorly-fed without acetate

supplement (PFA⁻), and poorly-fed with acetate supplement (PFA⁺). Because 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 ($P \leq 0.05$; Table 1)

than poorly-fed individuals, regardless of sex or presence of acetate supplement (Fig. 1, Table 1). However, even well-fed individuals were unable to replenish their defenses to levels observed before the experiment (Fig. 1). There was no significant effect of sex or presence of acetate supplement on the mass or concentration of total benzoquinones (Table 1).

Discussion

Although chemical defenses are widespread among arthropods, representing one of the main lines of defense against predators (Whitman et al. 1994), little attention has been devoted to investigate how the production of defensive compounds responds to condition. Here, we designed an experiment to address this subject, and showed that well-fed individuals of the harvestman *M. neptunus* produce secretions with higher mass and concentration of benzoquinones than poorly-fed individuals. This finding supports the hypothesis

that the production of chemical defenses depends on the amount of food available to the individuals.

Recently, we showed that non-ovigerous females of the harvestman *Acutisoma longipes* (Gonyleptidae) produce 72 % more defensive secretions than ovigerous females, probably as the result of allocation trade-offs between chemical defenses and egg production. Although the concentration of benzoquinones produced by ovigerous and non-ovigerous females did not differ, we showed that the greater amount of secretions released by non-ovigerous provides more effective protection against predators (Nazareth and Machado 2015). Here, we showed that poorly-fed individuals of *M. neptunus* produce defensive secretions with lower mass and concentration of benzoquinones, thus they should also be more vulnerable to predation. In this species, males care for eggs, and during the long period of offspring attendance, the feeding frequency of the males is markedly reduced compared to males not tending the offspring (Nazareth and Machado

Table 1 Results of the repeated measures analyses of variance considering the effects of time (before and after the experiment), sex (males and females), food availability (well-fed and poorly-fed), acetate supplement (present and absent), and their interactions on the mass andconcentration of total benzoquinones produced by individuals of the harvestman *Magnispina neptunus* (df = degrees of freedom, MS = mean squares, F = F -statistic, and P = P -value). Significant effects are highlighted in bold

Source of variation	df	Mass			Concentration		
		MS	F	P	MS	F	P
Between subjects							
Sex	1	1.961	1.05	0.309	2.165	1.366	0.246
Food	1	4.614	2.47	0.12	3.117	1.967	0.164
Acetate	1	3.886	2.08	0.153	1.897	1.197	0.277
Sex* Acetate	1	1.209	0.647	0.424	1.372	0.866	0.355
Sex* Acetate	1	1.17	0.626	0.431	1.028	0.649	0.423
Food * Acetate	1	0.013	0.007	0.934	0.122	0.077	0.782
Sex* Food * Acetate	1	0.164	0.088	0.768	0.052	0.033	0.857
Error	83	1.868			1.585		
Within subjects							
Time	1	8.979	7.430	0.008	29.414	27.893	<0.001
Time* Food	1	19.02	15.738	<0.001	6.588	6.247	0.014
Time* Acetate	1	0.001	0.001	0.973	0.482	0.457	0.501
Time * Sex	1	0.093	0.077	0.782	0.326	0.309	0.580
Time* Food * Acetate	1	0.767	0.635	0.428	0.059	0.056	0.814
Time* Food * Sex	1	0.009	0.008	0.93	0.014	0.014	0.908
Time* Acetate * Sex	1	0.958	0.792	0.376	0.76	0.721	0.398
Time* Food * Acetate * Sex	1	0.432	0.357	0.552	0.353	0.335	0.565
Error	83	1.209			1.055		

2010). Food deprivation as a result of paternal behavior may compromise chemical defenses and increase the likelihood of predation.

An acetate-supplemented diet does not improve the production of defensive secretions in *M. neptunus*. Unfortunately, our knowledge of harvestman metabolism is limited. It is not clear whether the enzymes for the polyketide pathway are produced by harvestmen themselves or whether the enzymes are acquired from symbiotic microorganisms. Genes for polyketide synthases have been reported for bacteria, fungi, algae, and plants, but there is no evidence that such genes exist in insects (Pankewitz and Hilker 2008). If the polyketide pathway in harvestmen depends on symbiotic microorganisms, the production of benzoquinones may not be strictly related to the amount of acetate acquired by the individuals. Even if the enzymes are endogenous rather than acquired, food limitation may intensify allocation trade-offs. For example, poorly-fed individuals may direct all acetate to the production of vital molecules, including fatty acids and glucose (via gluconeogenesis). Conversely, if well-fed individuals have sufficient metabolic reserves, the acetate supplement should not substantially improve the production of chemical secretions, as we found here. However, this possibility is unlikely because the amount of benzoquinones released

at the end of the experiment was lower than at the first extraction even for well-fed individuals.

In summary, the production of alkylated benzoquinones by the harvestman *M. neptunus* is condition-dependent, indicating that chemical defenses are a costly trait. The main implication of this finding is that even short periods of dietary restriction, like the one in this study, may make individuals more vulnerable to predators, imposing possible fitness consequences to chemically-protected arthropods that need to biosynthesize their own defensive compounds.

Acknowledgments We thank S. Rodrigues and R. Crespo for logistical support at LaReMN-UFF, A.H. Januário, A. González, M.F.K. Becker, B.A. Buzatto, N. Chaline, A. Arab, and two anonymous reviewers for comments on the manuscript, and FAPESP (2011/50800-8, 2012/50229-1) and CNPq (306550/2014-8) for financial support.

References

- Bonduriansky R (2007) The evolution of condition-dependent sexual dimorphism. *Am Nat* 169:9–19
- Gnaspini P, Hara MR (2007) Defense mechanisms. In: Pinto-Da-Rocha R, Machado G, Giribet G (eds) *Harvestmen: The biology of Opiliones*. Harvard University Press, Cambridge, pp. 374–399

- Kogel S, Eben A, Hoffmann C, Gross J (2012) Influence of diet on fecundity, immune defense and content of 2-isopropyl-3-methoxypyrazine in *Harmonia axyridis* Pallas. *J Chem Ecol* 38:854–864
- Nazareth TM, Machado G (2010) Mating system and exclusive postzygotic paternal care in a Neotropical harvestman (Arachnida: Opiliones). *Anim Behav* 79:547–554
- Nazareth TM, Machado G (2015) Egg production constrains chemical defenses in a Neotropical arachnid. *PLoS One* 10:e0134908
- Palottini F, González A, Manrique G (2014) Filling dynamics of the Brindley's glands in the blood-sucking bug *Triatoma infestans* (Hemiptera: Reduviidae). *J Insect Physiol* 71:122–127
- Pankewitz F, Hilker M (2008) Polyketides in insects: ecological role of these widespread chemicals and evolutionary aspects of their biogenesis. *Biol Rev* 83:209–226
- Rocha DFO, Wouters FC, Zampieri DS, Brocksom TJ, Machado G, Marsaioli AJ (2013) Harvestman phenols and benzoquinones: characterization and biosynthetic pathway. *Molecules* 18:11429–11451
- Rowe L, Houle D (1996) The lek paradox and the capture of genetic variance by condition dependent traits. *Proc R Soc Lond B* 263:1415–1421
- Whitman DW, Blum MS, Alsop DW (1994) Allomones: Chemicals for defense. In: Evans DL, Schmidt JO (eds) *Insect defenses: Adaptive mechanisms and strategies of prey and predators*. State University of New York, Albany, pp. 289–351