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Does the presence of Ethanol and Flunitrazepam (Rohypnol®) influence the attractiveness of scavenger insects to carcasses? A pilot study in an Atlantic Forest fragment

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Resumo

Flunitrazepam e etanol são frequentemente envolvidos em crimes, embora estudos investigando os efeitos do pré-tratamento com ambas as substâncias na atratividade de carcaças de vertebrados para insetos necrófagos sejam escassos. Neste estudo, objetivamos investigar o efeito do etanol, flunitrazepam e sua ação combinada na composição e estrutura de assembleias de dípteros visitantes, bem como no padrão de sucessão da fauna cadavérica que pode auxiliar em investigações forenses. Para isso, 32 ratos Wistar fêmeas foram igualmente divididos em quatro grupos com oito ratas cada: I - água (controle), II - etanol (21 dias, única fonte de líquidos), III - flunitrazepam (dose única, via oral, 2 mg/kg) e IV - etanol (21 dias, única fonte de líquidos) mais flunitrazepam (dose única, 2 mg/kg). As carcaças dos animais foram colocadas em 32 armadilhas dispostas dentro de uma área protegida e os insetos visitantes foram monitorados e coletados durante cinco dias consecutivos. Um total de 3.165 dípteros necrófagos das famílias Calliphoridae (61,7%), Fanniidae (10,5%), Sarcophagidae (10,4%), Anthomyiidae (4,0%), Muscidae (2,2%) e Phoridae (0,9%) foram coletados. A maior taxa de atração de insetos foi observada no tratamento combinado de etanol e flunitrazepam (44,3% dos espécimes), em comparação com os tratamentos de etanol (20,8%) e flunitrazepam (15,0%), e grupos de controle (19,7%). A composição e estrutura das assembleias são mais semelhantes em carcaças com etanol. Além disso, a presença de etanol e tratamento combinado alterou o padrão de sucessão dos insetos. Esses resultados indicam que o uso combinado de ambas as substâncias relacionadas ao estupro aumenta a atratividade de carcaças para insetos, o que poderia ser um sinal entomológico crucial encontrado em investigações criminais.

Palavras-Chave: Entomologia Forense, inseto necrófago, droga para estupro, carcaça.

Abstract

Flunitrazepam and ethanol are frequently involved in crimes, although studies investigating the effects of pretreatment with both substances on the attractiveness of vertebrate carcasses to necrophagous insects are scarce. In this study, we aimed to investigate the effect of ethanol, flunitrazepam and its combined action on the composition and structure of assemblies of visiting dipterans, as well as on the succession pattern of cadaverous fauna that can assist on forensic investigations. For that, 32 female Wistar rats were equally divided into four groups with eight rats each: I - water (control), II - ethanol (21 days, only source of fluids), III - flunitrazepam (single dose, oral route, 2 mg/Kg) and IV - ethanol (21 days, only source of fluids) plus flunitrazepam (single dose, 2 mg/Kg). Animals carcasses were placed in 32 traps set within a protected area and the visiting insects were monitored and collected for five consecutive days. A total of 3,165 necrophagous dipteran from the families Calliphoridae (61.7%), Fanniidae (10.5%), Sarcophagidae (10.4%), Anthomyiidae (4.0%), Muscidae (2.2%), Phoridae (0.9%) were collected. The highest rate of insect's attraction was observed in the ethanol plus flunitrazepam combined treatment (44.3% of specimens), compared with the ethanol (20.8%) and flunitrazepam (15.0%) treatments, and control groups (19.7%). The composition and structure of the assemblies are more similar in carcasses with ethanol. Besides, the presence of ethanol and combined treatment altered the pattern of insect's succession. These results indicate that the combined use of both rape-related substances increases the attractiveness of carcasses for insects, which could be a crucial entomological signal found in criminal investigations.

Keywords: Forensic Entomology, necrophagous insect, date rape drug, carrion.

1. INTRODUCTION

Forensic Entomology uses insects and other arthropods as diagnostic tools for the elucidation of a wide variety of questions in criminal and civil law [1-4]. In violent death criminal investigations, insects provide important diagnostic tools given their dispersal capacity and attraction to decomposing biomass, which provides a temporary microhabitat for feeding and reproduction. Insects are the first to arrive in a dead body and are present throughout the decomposition process [5]. The systematic evaluation of a corpse attractiveness permits the reliable identification of the associated insect fauna and the composition of the visiting community, representing an optional way to estimate the time of death, also known as post-mortem interval (PMI) [3,5].

However, the accuracy of calculating PMI based on entomological data can be significantly altered in cases of deaths involving narcotic intoxication [3,6]. Entomotoxicology thus emerges as a crucial research area for analyzing the influence of chemical substances on the assembly of scavenger insects associated with decomposing carcasses [6], contributing to correct determination not only of PMI, but also of drug detection and its association with the individual's cause of death.

There is a lack of information about the attractive and repellent role of chemicals such as ethanol or flunitrazepam in the dynamic of insects of forensic importance's attractiveness to decomposing corpses. Both these substances are socially important given their frequent and often abusive consumption. Ethanol (alcohol) is an addictive psychoactive substance [7] and represents the most frequently detected chemical in many postmortem cases [8,9]. In addition, it is cheap and easily obtained, which make their excessive intake a major role in serious accidents and trauma related deaths [10,11].

On the other hand, flunitrazepam (Rohypnol®) is a benzodiazepine drug used as a sedative, pre-anesthetic and in the treatment of insomnia, in which it acts by depressing the central nervous system [12]. Since is a psychotropic drug, its accidental or illicit ingestion is generally associated with cases of sexual assault and rape cases, where is known as the "date rape drug" [13], in which it is used to induce a rapid and profound state of unconsciousness and subsequent amnesia.

Many crimes are committed with the association of both ethanol and flunitrazepam due to the fact that benzodiazepine and ethanol co-administration affects the nervous system and impairs cued recall [13,14,15]. This study aims to evaluate how ethanol, flunitrazepam and their combined action interferes with the composition and structure of assemblies of visiting dipterans in intoxicated carcasses, as well as in the patterns of succession of cadaverous fauna which can assist in investigative processes of drug-associated death.

2. MATERIAL AND METHODS

2.1. Study area

The field experiment was conducted at the 7th Battalion of Army Engineers in Natal (5°49'50.2" S, 35°11'41.6" W) in the state of Rio Grande do Norte, Brazil. This area is part of the Jornalista Luis Maria Alves State Park (Natal Dunes State Park), a conservation unit that protects the local Atlantic Forest biome. The Natal Dunes State Park is the second largest urban park in Brazil, and has a total length of 9 km, with an area of 1172.80 ha of coastal dune habitats dominated by different morphoclimatic domains [16], such as the Atlantic Forest and Coastal Plateau [17]. The area is located at the northern extreme of the Brazilian Atlantic Forest, and is surrounded by urban development, with widespread evidence of anthropogenic impact.

2.2. Acquisition and preparation of experimental animals

A total of 32 female Wistar (*Rattus norvegicus* – Berkenhout, 1769) rats, with a mean weight of 255±30 g, were used in the trials. All experiments were conducted in accordance with Brazilian law Nº 11.794/2008 for animal experimental use. The experimental procedures were pre-approved by the Ethics Committee, through protocol number 044/2013. This study is reported following the ARRIVE guidelines [18].

The rats were equally divided into four different treatments groups, with eight rats per groupt: (I) control – offered only water, ad libitum; (II) ethanol – ethyl alcohol (Alcoolabor®, Segmenta) administered at increasing doses of 2% over 3 days, 4% (3 days), and 6% (15 days) over a 21-day period [19,20]; (III) flunitrazepam – single dose of 2 mg/kg taken orally [21]; (IV) ethanol plus flunitrazepam – combined dose of the two substances, as described in the previous two treatments [21]. One hour after flunitrazepam administration, the rats were euthanized by decapitation using a guillotine. The animals had free access to laboratory chow Purina, and water until the day of euthanasia and setting up of the experiment.

Each rat was then placed in a sealed plastic bag for transportation and isolated by group. In the field, they were individually disposed in suspended traps adapted from Ferreira [22]. The traps were made of PET bottles, with a side opening of approximately 10 cm to allow insects entering, and a second opening in the upper part of the trap, where a collection bag was placed. The traps were distributed in eight grids located along the trail of the 7th Battalion of Army Engineers, at approximately 50 m intervals, at 1.50 meters above the ground. Each grid contained one rat of each treatment (control, ethanol,

flunitrazepam, and combined), separated by a distance of 5 meters. The traps remained in the field throughout five consecutive days in the dry season. This period was determined due to the rapid decomposition of the carcasses, especially in the dry season.

2.3. Monitoring of the traps

After 24 hours of decomposition, the traps were checked daily at 09:00 h. The monitoring consisted of the removal of the plastic bags containing adult insects, which were replaced by new bags. The collected bags were stored in Styrofoam boxes, separated by treatment and transported to the Laboratory of insects and vectors (LiVe-UFRN) for identification using dichotomous species keys [23,24]. Meteorological data (relative humidity, temperature, wind speed, and precipitation) were obtained from the Principal Climate Station of the UFRN Geography Department in Natal.

2.4. Statistical analyses

The relation between the four treatments (ethanol, flunitrazepam, combined, and control) was analyzed, as well as the diversity of insect species and families, and the days of the study period.

An Analysis of Covariance (ANCOVA) was used to evaluate the influence of the four treatments on the insect species/families collected during the different days of the study. This analysis was run in XLSTAT 2015. The analysis of the four treatments in relation to the similarity of the species collected was based on a Non-Metric Multidimensional Scaling (nMDS) procedure, which reflects the similarity among the samples, using a matrix of fly species abundance. This test provides graphic displays showing the clustering of the more similar treatments, with more distinct groups being separated by wider distances [25].

For this analysis, the abundance data for each treatment were log (x+1) transformed, and the Bray-Curtis similarity index was used to evaluate the similarity between the four treatments (ethanol, flunitrazepam, combined, and control). In addition to nMDS, we used the Hierarchical Cluster analysis to access the similarity index between treatments for both structure and composition of assemblies. The nMDS analysis and Hierarchical Cluster analysis was run in Primer® 6.0. To evaluate the interference of environmental variables on insect abundance, a multiple linear regression was run in BioEstat® 5.3.

3. RESULTS

3.1. General aspects of the insect assembly

A total of 3,148 necrophagous dipterans were collected during the five days of sampling, most of which belonged to the family Calliphoridae (62.1%), followed by Fanniidae (10.5%), Sarcophagidae (10.4%), Anthomyiidae (4.0%), Muscidae (2.2%) and Phoridae (0.9%) (Tab. 1). Interestingly, 9.5% of the adults collected represented wasps, and of these, approximately 38% were collected in the combined group, followed by the flunitrazepam (29%), alcohol (20%) and control (13%) group (Tab. 1).

Species richness was higher for Calliphoridae and Muscidae, with a record of 5spp each, followed by Sarcophagidae (4spp), Fanniidae (2spp) and Phoridae, with one specie (Tab. 1). Among the registered species, *Chrysomya megacephala* (Fabricius, 1794) was the most abundant, with 38.7% of the collected specimens, followed by *Chrysomya albiceps* (Wiedemann, 1819) (17.6%), *Chrysomya putoria* (Wiedemann, 1818) (2,7%) and *Lucilia eximia* (Wiedemann, 1818) (2.5%) (Fig. 1B).

For Fanniidae, only specimens of the genus Fannia were identified, with emphasis on *Fannia pusio* (Wiedemann, 1830). On the other hand, for Muscidae, the species *Hydrotaea aenescens* (Wiedemann, 1830) and *Atherigona orientalis* (Schiner, 1868) were the most abundant (Tab. 1). *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) was the most representative sarcophagid, with the other representatives registered in low abundance and belonging to the genus *Oxysarcodexia* (Tab. 1).

In general, insect assemblies were not influenced by the meteorological variables ($P > 0.05$). Mean temperature was 26.3 °C, varying from 25 °C to 28 °C. Relative humidity ranged from 78% to 94%. There was 12 mm of precipitation on the second day of the experiment, with no precipitation being recorded on the first and fourth days, and an overall average of 3.92 mm. Wind speeds were between 3.3 km/h and 5.9 km/h.

3.2. Effect of treatments on the assembly of dipterans and entomological succession

Regarding treatments, we found that the combined treatment was more attractive than the others ($P < 0.05$), which concentrated 44.2% of all collected visitors ($P < 0.05$), followed by ethanol (20.8%), control (19.7%) and flunitrazepam (15.0%) (Fig. 1A). The abundance also varied according to the decomposition time, with days 2 (48h), 3 (72h), 4 (96) and 5 (120h) more representative than day 1 (24h) ($P < 0.05$). In the other hand, there was a variation between the number of species recorded between the groups, with 10spp in the control, Flunitrazepam and Combined, and 15spp in the Ethanol group.

Table 1. The abundance of dipterans collected on rat carcasses submitted to control, ethanol, flunitrazepam, and combined treatments, in an area of Atlantic Forest in the municipality of Natal, Brazil.

Family/Species	Control	Ethanol	Flunitrazepam	Combined	Total	%
Calliphoridae	**377**	**373**	**202**	**1,003**	**1,955**	**62**
Chrysomya megacephala (Fabricius, 1794)	288	183	121	633	1,225	38,7
Chrysomya albiceps (Wiedemann, 1819)	71	151	43	294	559	17,6
Chrysomya putoria (Wiedemann, 1818)	16	14	11	47	88	2,7
Lucilia eximia (Wiedemann, 1818*)*	2	24	26	28	80	2,5
Cochliomyia macellaria (Fabricius, 1775)	0	1	1	1	3	0,1
Sarcophagidae	**71**	**70**	**73**	**116**	**330**	**10**
Peckia (Peckia) chrysostoma (Wiedemann, 1830)	1	4	3	4	12	0,3
Oxysarcodexia timida (Aldrich, 1916)	1	0	1	0	2	0,1
Oxysarcodexia amorosa (Schiner, 1868)	0	1	0	0	1	0,1
Oxysarcodexia intona (Curran & Walley, 1934)	0	4	0	0	4	0,1
*Sarcophagidae sp**	69	61	69	112	311	0,6
Muscidae	**14**	**40**	**6**	**11**	**71**	**2,2**
Musca domestica (Linnaeus, 1758)	3	1	1	0	5	0,1
Hydrotaea aenescens (Wiedemann, 1830)	2	30	0	7	39	1,2
Hydrotaea chalcogaster (Wiedemann, 1824)	1	0	0	0	1	0,1
Atherigona orientalis (Schiner, 1868)	4	8	1	4	17	0,5
Synthesiomyia nudiseta (Wulp, 1883)	0	1	0	0	1	0,1
Fanniidae	**83**	**70**	**74**	**106**	**333**	**11**
Fannia pusio (Wiedemann, 1830)	0	3	2	1	6	0,1
Fannia sp.	0	25	0	9	34	1
Phoridae	**6**	**13**	**4**	**8**	**31**	**0,9**
Megaselia scalaris (Loew, 1866)	0	1	0	0	1	0,1
Anthomyiidae	**34**	**29**	**21**	**43**	**127**	**4**
Wasps	**40**	**61**	**87**	**113**	**301**	**9,5**
Total	**619**	**656**	**467**	**1,392**	**3,148**	**100**

* Females from the family Sarcophagidae

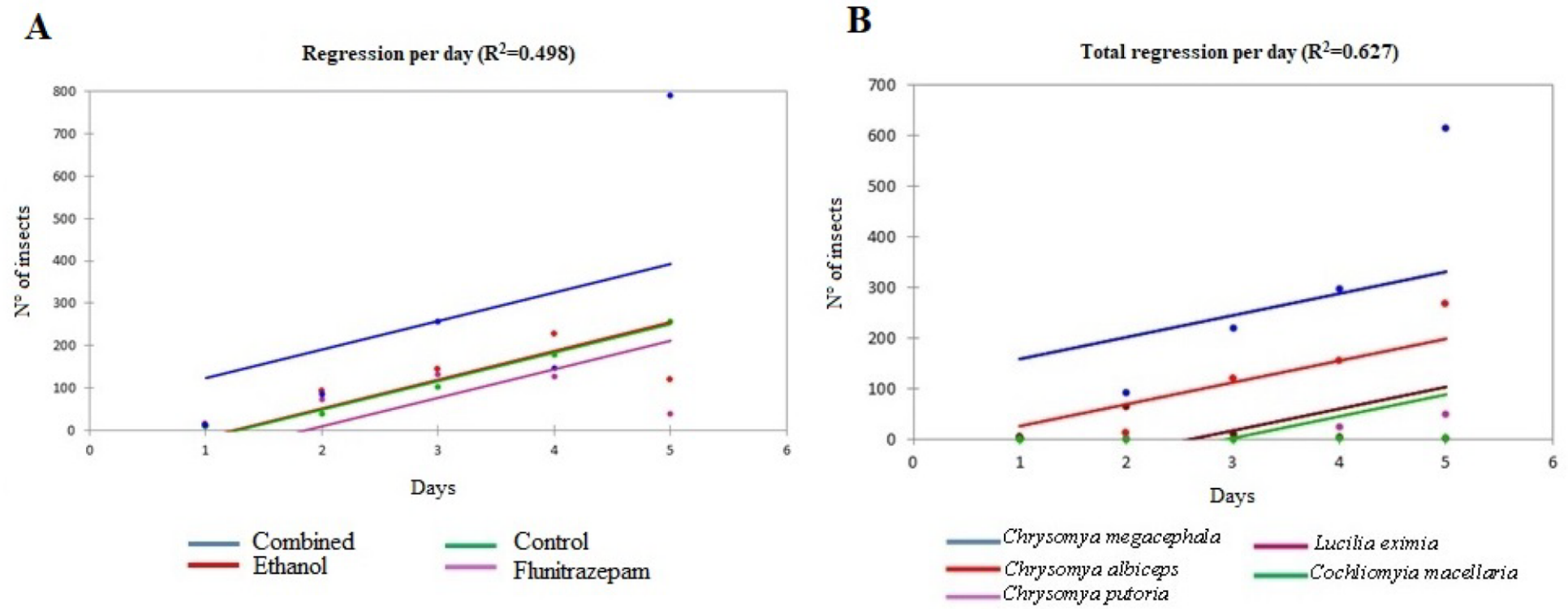

Figure 1. Results of the ANCOVA for **A)** the dipterans collected on rat carcasses in the control, ethanol, flunitrazepam, and combined treatments in an area of Atlantic Forest in the municipality of Natal, Brazil; and **B)** the Calliphoridae species collected on rat carcasses in the control, ethanol, flunitrazepam, and combined treatments in an area of Atlantic Forest in the municipality of Natal, Brazil.

The calliphorids and sarcophagids presented increasing abundance during the course of the study period, except in the case of *L. eximia*, which peaked on the second day, decreasing abruptly in the subsequent three days (Fig. 2). *Cochliomyia macellaria* (Fabricius, 1775) was collected only on the fourth and fifth days of the study. Wasps presented a greater abundance on days 1 and 2 (Fig. 2).

The dynamics of the attractiveness of necrophagous dipterans in all four treatments during the course of the study period can be observed in Tab. 2 and it shows that the presence of only ethanol anticipated by one day the arrival of all species of the genus *Chrysomya* and individuals of the family Muscidae, Phoridae and Fanniidae when compared to the control. When analyzed alone, flunitrazepam had no significant effect on the attractiveness of visiting insects (Tab. 2). However, when associated with ethanol, it delayed the attractiveness of *C. putoria* by two days when compared to ethanol group. This group anticipated the arrival of Phoridae by one day when compared to the ethanol and control group, and two days with respect to flunitrapezam treatment.

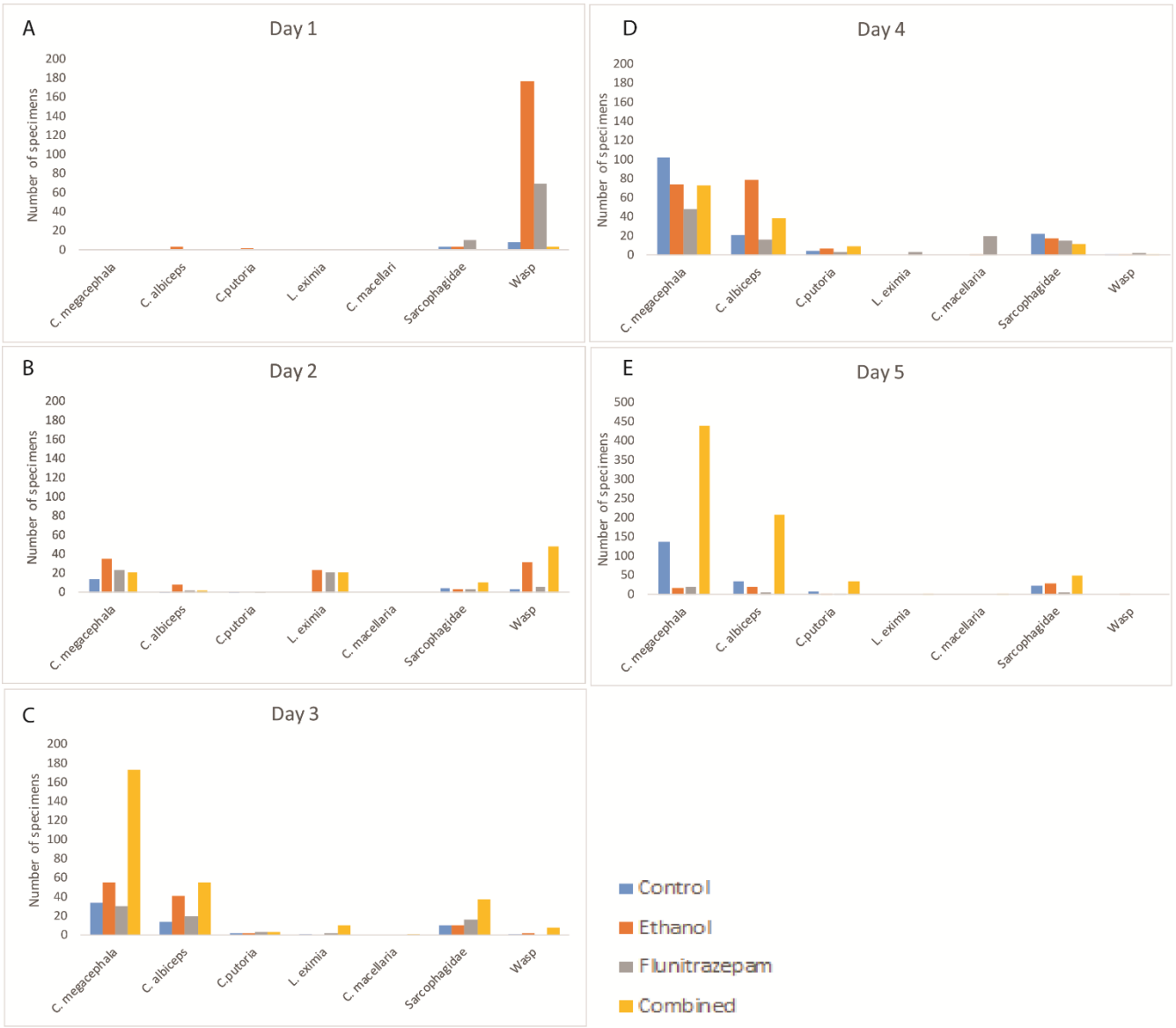

Figure 2. Distribution of species collected in the control, ethanol, flunitrazepam and combined groups following the experimental days. A) day 1; B) day 2, C), day 3, D) day 4 and E) day 5 of collection.

Thus, the presence of ethanol combined with flunitrazepam alters the arrival pattern of visiting insects in the carcasses when compared to control and ethanol intoxicated rats, except for the *Sarcophagidae* family. This

family showed the same pattern of entomological success in all treatments (Tab. 2)

The necrophagous insects were distributed heterogeneously among treatments. The multivariate (nMDS) analysis of the abundance of insects in relation to the factor "drugs" (presence of ethanol and/or flunitrazepam) found no clear ordination of the treatments (Fig. 3). While attractiveness increased over the five days of the study period, there was no clear tendency for any grouping of the replicates among treatments on the same day. However, according to the Hierarchical Cluster analysis results, we observed that the composition and structure of the assemblies between treatments with ethanol are more similar (Fig. 4).

Table 2. Succession of insects collected in rat carcasses from control, ethanol, flunitrazepam and combined treatments, in an Atlantic Forest area of, the municipality of Natal, in the state of Rio Grande do Norte, during the experiment. Values referring to eight replicates for each treatment. ●= 0-10 ●● = 11 -50 ●●● = 50 -100 ●●●● = 101-150

Species	Control					Ethanol					Flunitrazepam					Combined				
	Days					Days					Days					Days				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
C. megacephala		●	●	●●●	●●●		●	●●	●●	●		●	●	●	●		●	●●	●●	●●●
		●	●	●	●	●	●	●	●	●		●	●	●	●		●	●	●	●
			●						●●	●			●	●						●●●
C. albiceps		●	●	●●	●●	●	●	●●	●	●		●	●	●	●		●	●●	●●	●
C. putoria		●	●	●	●	●		●	●	●		●	●	●	●			●	●	●●
							●					●					●			
Lucilia eximia	●		●			●	●					●	●	●			●	●		●
Co. macellaria									●					●						●
			●							●	●		●	●			●			
Sarcophagidae	●	●	●	●●	●●	●	●	●●	●●	●	●	●	●	●	●	●	●	●●	●●	●●
		●					●			●										
Anthomyiidae	●	●		●		●	●		●	●	●	●	●	●	●	●	●	●	●	●●
										●										
Muscidae		●	●	●	●	●		●	●●	●		●	●	●	●		●	●	●	●
			●							●		●	●	●			●	●●		
Fanniidae			●	●●	●●		●	●●	●●	●		●	●	●			●	●	●	●●
Phoridae			●	●	●		●	●		●			●		●	●		●	●	●

4. DISCUSSION

The accidental or illicit ingestion of flunitrazepam (Rohypnol®) is generally associated with cases of sexual assault and rape, where is known as the "date rape drug" [13]. The use of flunitrazepam in combination with alcohol is of forensic particular concern as both are central nervous system depressants and may potentiate each other's toxicity [26], leading to loss of memory and death [27]. Is this regard, it became of great importance the study of the chemical attractiveness or repellence of this chemical combination to the necrophagous fauna associated with carcasses decomposition, representing an important investigative entomotoxicological tool [28]. In this work, we could observe that the animals that received ethanol and flunitrazepam registered approximately 45% of all insects attracted to the carcasses, of which 71% correspond to calliphorids. Besides, this combination changed the Diptera visitation pattern, being able to assist in the future investigative processes with suspicion of rape related drug use.

Decaying carcasses and corpses can attract large numbers of organisms from which arthropods constitute the predominant fauna [2,3]. This study showed that the rat carcasses were visited and decomposed by a wide diversity of necrophagous insects (Tab. 1), dipterans of the families Calliphoridae, Fanniidae, and Sarcophagidae.

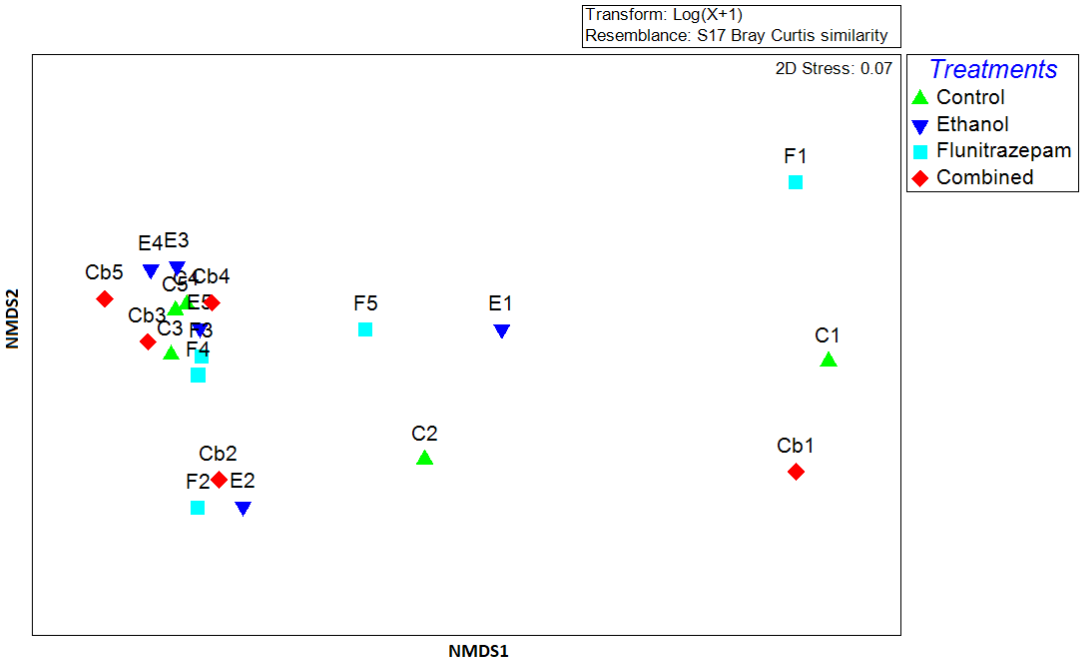

Figure 3. Distribution of the four treatments based on the abundance of insects collected during the five days of the experiment, based on a Non-Metric Multidimensional Scaling (nMDS) approach. The letters correspond to the treatments and the numbers represent the days.

This data reflects the role of the carcass as a basic substrate for the colonization and development of insects [29], which contribute to the process of decomposition, and are valuable diagnostic cues for forensic studies [3].

Interestingly, the alcohol group was the second most abundant of the groups studied, while flunitrazepam was the least attracted by insects to carcasses. The results demonstrate that insects are attracted by the presence of alcohol, which was corroborated by the high similarity of alcohol and combined treatments (alcohol + flunitrazepam) as shown in fig. 4. The high abundance in carcasses with the presence of alcohol may be associated with the fact that this compound is a natural product of the decomposition process, which is commonly generated from tissue degradation by microorganisms such as *Candida albicans*, *Clostridium* sp., *Escherichia coli*, *Streptococcus faecalis* and *Lactobacillus* sp. [30,31].

In the United States, Tabor et al. [32] also evaluated the effects of the ante mortem ingestion of ethanol in pigs on the succession of insects and found that the pigs that ingested ethanol attracted more dipterans and coleopterans than those that had not ingested this substance. It is important to note that only three (*C. macellaria*, *Musca domestica* Linnaeus, 1758 and *Synthesiomya nudiseta* (Wulp, 1883)) of the 32 species identified in this study were also recorded in the present study, emphasizing the ecological differences between regions and the need to evaluate local patterns.

Our entomological data also corroborate with Goff and Lord [6], when they discussed that the presence of certain concentrations of toxins or drugs in a decomposing carcass, can alter the rate of invasion and attraction of decomposing insects. However, no data are available on the effects of ethanol and flunitrazepam olfactory stimuli on the behavior of necrophagous dipterans; although the results show that the combination of these substances attracts more insects than other treatments. This indicates that during a forensic investigation such as a rape facilitated by the use of recreational drugs and alcoholic beverages, the body of the victim may attract a larger quantity of necrophagous insects than it would in the absence of these substances. So, the relative abundance of insects, in this case, may contribute to the determination of the cause of death.

It is unclear what mechanisms determine the possible synergism between the ethanol and flunitrazepam for the necrophagous flies. However, the literature shows that after chronic administration of alcohol followed by a single dose of benzodiazepine, the enzymes of the Cytochrome P450 complex responsible for the metabolization of these drugs are induced and the demethylation and hydroxylation of different benzodiazepines is increased [33,34].

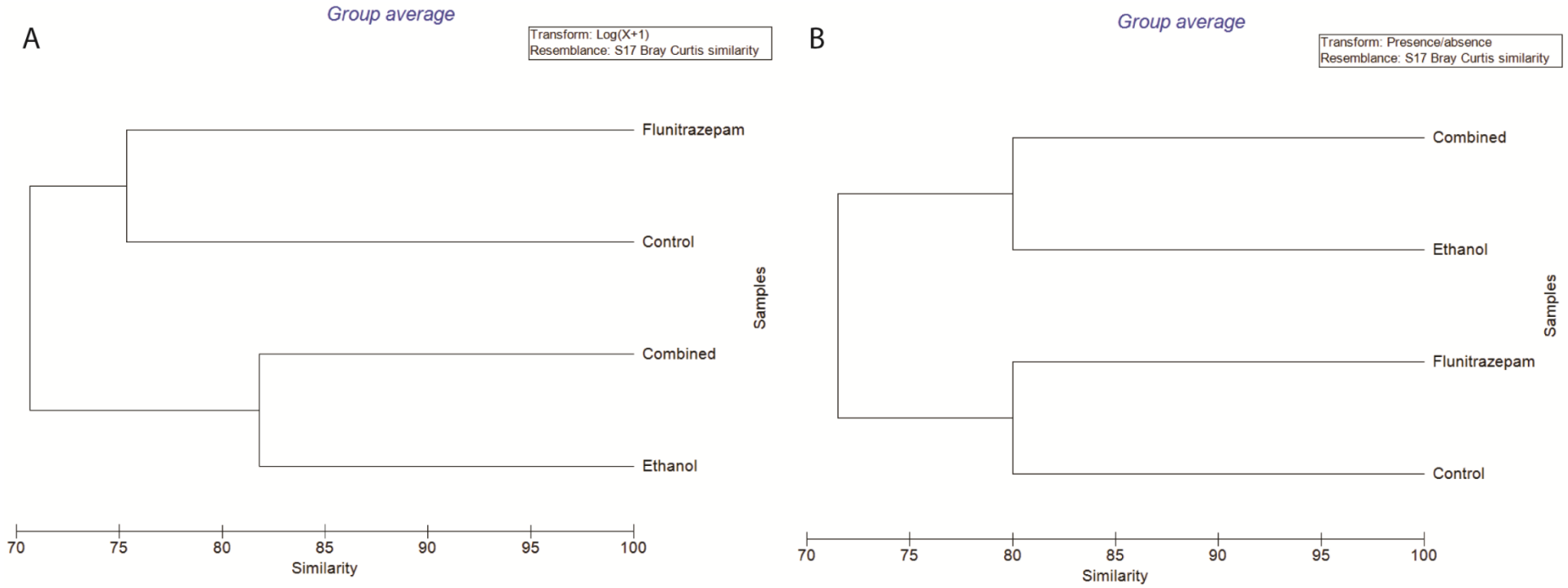

Figure 4. Similarity analysis (cluster dendrogram) of the necrophagous dipteran's assemblage associated with rat carcasses in the four treatments. A) Structure, that is, greater emphasis on the abundance of species in the samples, and B) for species composition.

Thus, one of the mechanisms that may be involved in increasing the attractiveness of scavenger insects is the rapid metabolism of flunitrazepam and the association of its reduced secondary metabolites with volatile compounds derived from alcohol metabolism in the liver, generating plumes of odors different from unintoxicated carcasses and more attractive to associated insects.

Takiguchi et al. [14] empirically demonstrated that the potentiated effect of benzodiazepines with ethanol is primarily due to increased brain levels of benzodiazepines, which justifies their use in crimes. Additionally, benzodiazepines can influence the development of Calliphoridae species, where *Chrysomya albiceps* fed on animal tissues with the drug developed more rapidly [35]. Baia et al. [36] also observed that the presence of flunitrazepam increases the mortality of immature *Chrysomya megacephala*, indicating that drugs impact insect life cycles and should be considered when estimating the post-mortem interval (PMI).

Due to their acute olfaction, necrophagous dipteran are pioneers in the colonization of decomposing corpses, and remains throughout the decomposition process, as observed in the present study [2,3,37]. *Chrysomya megacephala* was the most abundant species, followed by *C. albiceps*, *C. putoria*, *L. eximia*, and *C. macellaria*. This abundance of specimens is probably due to the ecological characteristics of the calliphorids, which are cosmopolitan, due to their high dispersal capacity and ability to adapt to new environments [38]. These species are abundant on corpses in Brazil [39,40], and in many other countries, including Colombia [41], Italy [42] and Malaysia [43].

The dynamics of the attractiveness of necrophagous dipterans during the study shows that the succession pattern differs in the presence of ethanol alone or combined with flunitrazepam. The presence of only ethanol anticipated by one day the arrival of all *Chrysomya* species and individuals of the family Muscidae, Phoridae and Fanniidae when compared to the control, which has direct implications for the calculation of the post-mortem interval (PMI). Thus, if we consider that the presence of alcohol accelerates the location of the carcass by *Chrysomya* species, this may cause a preconceived colonization in relation to cases without the use of alcohol, although the PMI also depends on other factors, such as the effect of the substance on the development of immatures, which may be the target of future studies.

When analyzed alone, flunitrazepam had no significant effect on the attractiveness of visiting insects (Tab. 2). However, when associated with ethanol, it delayed the attractiveness of *C. putoria* by two days when compared to ethanol, which may cause a delay in the colonization of the substrate, and consequently a divergence in the PMI.

However, the presence of ethanol, flunitrazepam or both chemical stimuli combined, anticipated by two days the arrival of these necrophagous insects to intoxicated carcasses (Tab. 2) when compared to the control group, demonstrating a greater attractiveness of these compounds to scavengers. The combined group also anticipated the arrival of Phoridae by one day when compared to the ethanol and control group, and two days with respect to flunitrapezam treatment. Thus, the presence of ethanol combined with flunitrazepam alters the arrival pattern of visiting insects in the carcasses when compared to control and ethanol intoxicated rats.

Sarcophagids were present throughout the decomposition process and were the most common dipterans on the first day of the experiment. Four species were identified – *P. chrysostoma*, *Oxysarcodexia timida* (Aldrich, 1916), *Oxysarcodexia amorosa* (Schiner, 1868) and *Oxysarcodexia intona* (Curran and Walley, 1934) – although most specimens were identified only to the level of family. In most cases, many of the specimens were female, which hampered the comparison between species, and no species predominated in the region. Previous studies in forensic entomology in Rio Grande do Norte have also recorded the presence of sarcophagids in human corpses, but did not identify the species [44,45].

Despite the attractiveness of the traps and the numbers of insects collected during the five days of the study, we emphasize the need to perfect the traps used in the study to reduce the escape and mortality ratio caused by the decomposing matter and the liquids produced by the biomass. Through the evaluation of the variation in attractiveness among the treatments, it was possible to conduct an initial inventory of the necrophagous insect fauna found in the Atlantic Forest, as well as confirming that the combined presence of ethanol and flunitrazepam attracts more insects than either substance separately and this data could contribute on entomological investigations of the rape-related drug use cases.

4. CONCLUSÕES

This work showed that the combined action of ethanol with flunitrazepam increases the attractiveness of necrophagous insects and alters their succession pattern on rat carcasses, representing important information in forensic investigations in cases of rape-related drugs use.

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