SCIENTIFIC NOTE

Trypanosomatid Prevalence in *Nezara viridula* (L.), *Euschistus heros* (Fabricius) and *Piezodorus guildinii* (Westwood) (Heteroptera: Pentatomidae) Populations in Northern Paraná, Brazil

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Neotropical Entomology 34(2):341-347 (2005)

Prevalência de Tripanossomatídeos em Populações de *Nezara viridula* (L.), *Euschistus heros* (Fabricius) e *Piezodorus guildinii* (Westwood) (Heteroptera: Pentatomidae) no Norte do Paraná

RESUMO - O complexo de percevejos da soja possui um grande potencial de dano, seu controle é difícil ao final da safra por suas elevadas densidades populacionais. Trabalhos relacionados com organismos entomopatogênicos em percevejos são interessantes devido a suas possibilidades como agentes de controle e porque eles podem ser agentes causais de doenças em criação massal. A prevalência de tripanossomatídeos foi determinada nas espécies mais importantes da cultura da soja, no Brasil, *Nezara viridula* (L.), *Euschistus heros* (Fabricius) e *Piezodorus guildinii* (Westwood). Os insetos foram coletados na soja e hospedeiros alternativos durante a entressafra. Gotas de hemolinfa de cada adulto foram montadas em lâminas e observadas ao microscópio; o número de percevejos com e sem flagelados foi registrado. Os flagelados encontrados nas populações de *P. guildinii* e *E. heros* foram menores que os observados em *N. viridula*. A maior prevalência dos tripanossomatídeos foi observada nos espécimes de *N. viridula* coletados na soja alcançando 30% de infecção em janeiro, 2001, e 44% prevalência em fevereiro de 2003. Em *P. guildinii* as máximas prevalências foram observadas em janeiro e fevereiro de 2003 (16%) e em 27 de julho de 2003 com 20% dos espécimes infectados. Os flagelados na hemolinfa de *E. heros* foram menos freqüentes (máxima prevalência de 8,7%), sugerindo que esta espécie é menos suscetível que *N. viridula* e *P. guildinii*. Embora os níveis de prevalência sejam mais elevados que os observados para fungos entomopatogênicos, os tripanossomatídeos não parecem ser eficientes reguladores das populações de percevejos de percevejos em curto prazo.

PALAVRAS-CHAVE: Inseto, percevejo, protozoa, flagelado

ABSTRACT - The stink bug complex in soybeans has a great potential to cause damage, their control has been difficult due to high population densities at the end of the season. Studies related to entomopathogenic organisms in stink bugs are interesting for their possibilities to be used as biological control agents and because they could be an etiological agent in mass rearing. We determined the prevalence of trypanosomatids in the most important species of the stink bug complex in soybean, from Brazil, *Nezara viridula* (L.), *Euschistus heros* (Fabricius) and *Piezodorus guildinii* (Westwood). Insects were collected in soybean and on alternate hosts during the intercrop season. Hemolymph drops from each adult were mounted on slides and observed under microscope; stink bug numbers with and without flagellates were recorded. The flagellates found in *P. guildinii* or *E. heros* populations were smaller than that found in *N. viridula*. The higher prevalence of the trypanosomatids was observed on soybean reaching a peak of 30% of the sampled *N. viridula* specimens in January, 2001, and 44% prevalence in February, 2003. The maximum prevalences (16%) in *P. guildinii* were observed in January and February, 2003 and on July 27, 2003 with 20% of the specimens infected. Flagellates in the hemolymph of *E. heros* were less frequent (maximum prevalence of 8.7%), suggesting that this species is less susceptible than *N. viridula* and *P. guildinii*. Trypanosomatid prevalence was higher than that observed for entomopathogenic fungi, however they seem to be inefficient control agents in short term condition.

KEY WORDS: Insect, stink bug, protozoa, flagellate

The stink bug complex in soybeans has great potential to cause damage (Corrêa-Ferreira & Panizzi 1999), and their populations can reach unusual levels, in some cases 20 to 25 individuals per soybean row meter. At the end of the growing season, stink bug control is difficult due to high population densities and some cases of insecticide resistance were recently reported (Sosa-Gómez *et al*. 2001). Although an egg parasitoid can be used (Corrêa-Ferreira 1993) most of the time stink bugs are controlled by a large spectrum of insecticides.

Studies related to entomopathogenic organisms in stink bugs are interesting because of their possibilities to be used as biological control agents and also because they could be a source of diseases during mass rearing. The most common naturally occurring pathogens of stink bugs are fungi [*Beauveria bassiana* (Bals.) Vuill., *Metarhizium anisopliae* (Metsch.) Sorok., *Paecilomyces fumosoroseus* (Wize) Brown and Smith], some RNA viruses (ssRNA small picorna-like virus and a dsRNA Toti-like virus) (Williamson and Wechmar 1992, 1995), and protozoan flagellates (Gibbs 1957, Batistoti 1998, Fuxa *et al*. 2000). However, a search for key natural control agents in stink bugs field populations has been neglected. Among the pathogens associated with soybean stink bugs, none of them cause perceivable epizootics. Sosa-Gómez & Moscardi (1998) reported high prevalence of *B. bassiana* on *Nezara viridula* (L.), in the greenhouse, an artificially protected environment, but cases like this have not been observed in the field.

Studies on 17 heteropteran species from Brazil revealed that 13 of them had trypanosomatid infections (Godoi 2000). Among the sampled specimens, two pentatomid species, *Euschistus* and *Edessa rufomarginata* (DeGeer) were not infected. Thus, although the presence o trypanosomatid in Brazilian stink bugs is known (Batistoti 1998, Godoi 2000) no information is available about protozoan prevalence in the more important stink bug pests of soybean [*Euschistus heros* (Fabricius), *N. viridula* e *Piezodorus guildinii* (Westwood)].

Therefore our purpose was to monitor these pathogens in the hemolymph of soybean stink bugs for three years. We determined the prevalence [see Fuxa and Tanada (1987)] of flagellates in the three more important species of the stink bug complex from Brazil, *N. viridula*, *E. heros* and *P. guildinii*.

Adult insects were collected in soybean fields during the summer and on alternate hosts during the intercrop season. *N. viridula* samples were obtained on chinese motherwort (*Leonorus sibiricus* L.) and wild radish (*Raphanus raphanistrum* L.), *P. guildinii* on indigofera (*Indigofera truxillensis* H.B.K. and *I. suffruticosa* Mill.) and pigeon pea (*Cajanus cajan* (L.) Millsp.), and *E. heros* in mulch of mango leaves (*Manguifera indica* L.) and on sunflower (*Helianthus annuus* L.). Collection sites were located in the North of Parana State: Warta, Guaravera (both from Londrina County), Jataizinho and Sertanópolis. Samples of stink bugs, ranging from 15 to 40 specimens of each species, were taken to the laboratory and bled through a shallow puncture on the lateral angle of the pronotum made with a stylet. The puncture was done inserting no more than 1 mm or 1.5 mm of the stylet tip inside the pronotal spine, far away enough of the digestive tract, to ensure that flagellates were not coming from the gut by the puncture. After gentle pressure, drops of hemolymph were collected on slides and observed with a phase-contrast microscope at 400 X; stink bugs with and without flagellates were recorded. The blood droplets were examined microscopically under the cover slip for the presence of flagellates, even though active forms were present in both hemolymph and feces. The droplet size was determined by weighting, assuming that the hemolymph have the same density of water. The flagellate bodies and flagella were measured with a OSM filar micrometer eyepiece (Ramsden type) (Olympus Optical Co. Ltd., Japan).

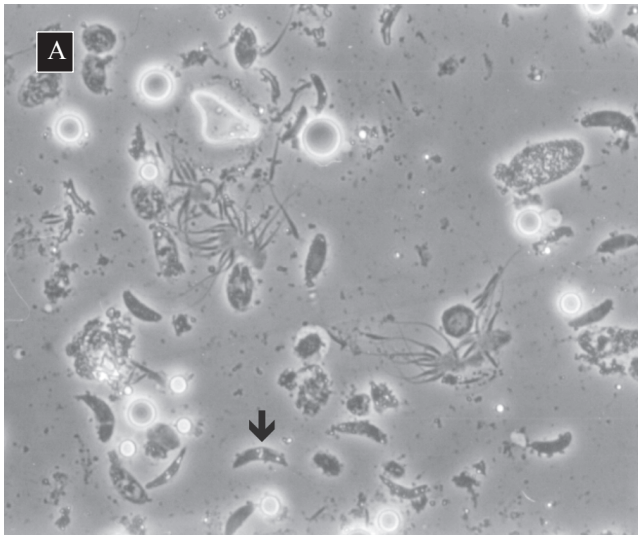
Because we did not initially detect flagellate infections in *E. heros* blood samples (n = 124), we suspected that this species was not susceptible to flagellatosis of the hemolymph. For this reason, *N. viridula* and *P. guildinii* samples were collected during 2000/2001, 2002/2003, and *E. heros* samples were collected starting from October 11, 2002 until August of 2003.

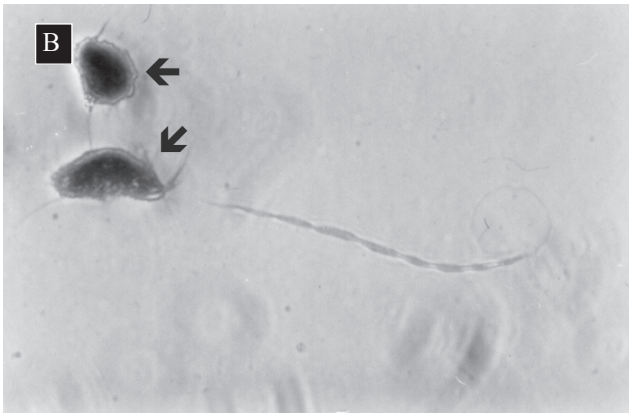
Comparisons of flagellates prevalence among stink bug species were done with the non-parametric test of Mann-Whitney Rank sum Test from Sigmastat Statistical Software (Jandel Scientific 1994).

Trypanosomatids were very common in *N. viridula* and *P. guildinii* populations (Fig. 1). Promastigotes and long forms were predominant. Flagellates found in *P. guildinii* and *E. heros* were smaller than those found in *N. viridula* (Table 1). Trypanosomatids from *N. viridula* were 50.1 ± 2.7 µm and the flagella were 31.5 ± 1.1 µm. One specimen found in *E. heros* had a body length of 25.1 µm and flagellum of 14.6 µm. In *P. guildinii*, the trypanosomatids were 22.9 ± 3.1 µm and 18.6 ± 3.1 µm, for body length and flagellum, respectively.

During the observational periods, flagellate prevalence increased in the populations of *N. viridula* and *P. guildinii* in the summer, during the wet and hot weather (January and/or February) reaching a peak of 30% in the sampled *N. viridula* specimens in January, 2001, and 44% prevalence in February, 2003 (Figs. 2 and 3). Therefore, we can consider that the rain distribution during the high prevalence period, could be positively related with the infection rates (Fig. 4). *N. viridula* had flagellates infection ranging from 2.5% to 44%, and *P. guildinii* had prevalence ranging from 2.9% to 20%. The maximum prevalences in *P. guildinii* were observed between January 29 and February 25, 2003 (16%) and on July 27, 2003 with 20% of the specimens infected. In *E. heros*, flagellates were observed on only three sampling dates in October, 2002, and only at prevalence rates of 3.3%, 8.7% and 4%. The prevalence in *E. heros* populations was significantly lower than in *N. viridula* populations ($P < 0.0001$, $T = 424.0$, $n = 17$, $N = 17$, Mann-Whitney Rank Sum Test) and *P. guildinii* populations ($P = 0.03$, $T = 382.5$, $n = 17$, $N = 19$, Mann-Whitney Rank Sum Test).

The average volume of hemolymph droplets obtained from *N. viridula* was higher (2.1 µl ± 0.7 (± SEM) than those from *P. guildinii* and *E. heros* (0.8 µl ± 0.4 and 0.5 µl ± 0.1, respectively). The number of flagellates per microliter of





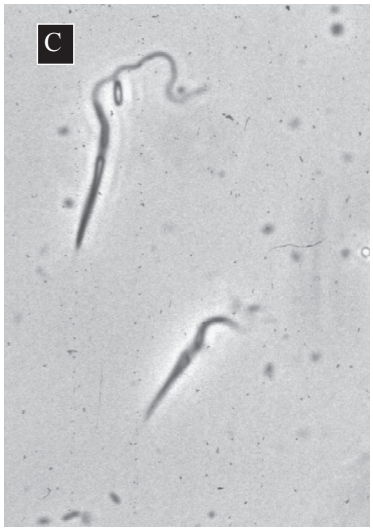


Figure 1. Phase-contrast image of flagellates: a) rosettes found in the hemolymph of *P. guildinii* and unidentified microorganisms (arrow); b) twisted form found in the hemolymph of *N. viridula* and hemocytes (arrows), from Londrina, PR, Brazil (c) in *N. viridula* from Quincy, Florida USA (note the cyst attached to the flagellum).

Table 1. Dimensions (µm) of flagellates occurring in pentatomid hosts.

	Body length	Body width	Flagella
N. viridula			
Mean ± SEM	50.1 ± 2.77	1.7 ± 0.14	31.5 ± 1.15
n	61	29	60
Range	22.1 - 113.1	0.5 - 3.4	17.4 - 52.8
P. guildinii			
Mean ± SEM	22.9 ± 3.14	1.5 ± 0.09	18.6 ± 3.08
n	35	19	19
Range	5.6 - 63.0	1.2 - 2.8	5.9 - 50.2

hemolymph in *N. viridula* (2.48 flagellates/µl) and *P. guildinii* (2.17 flagellates/µl) was not significantly different (P = 0.69, T = 781.0, n = 19, N = 67, Mann-Whitney Rank Sum Test) but was higher in *N. viridula* than in *E. heros* (0.50 flagellates/µl) (P < 0.009, T = 715.0, n = 22, N = 67, Mann-Whitney Rank Sum Test). No significant differences between *P. guildinii* and *E. heros* were observed. The number of trypanosomatids in the hemolymph of the three hemipterans ranged from 0 to more than 50 per blood sample (Fig. 5).

Although size differences were observed, morphological aspects are not conclusive to differentiate any taxon. Godoi (2000) stated that morphometric analysis was useless to resolve differencies among Genera. The taxonomy of this protozoan remains unclear at present; taxonomic studies of stink bug protozoans are necessary (Catarino *et al*. 2001). D.R. Sosa-Gómez & D.G. Boucias (unpubl.) observed infection by flagellates in *N. viridula* samples collected from corn during the summer of 1994, in Quincy, Florida, USA. Morphologically, the trypanosomatids from *N. viridula*, in the current study, are similar to those found in Louisiana (Fuxa *et al*. 2000) and to those that we found previously, from *N. viridula* collected from Quincy, FL. After isolation,

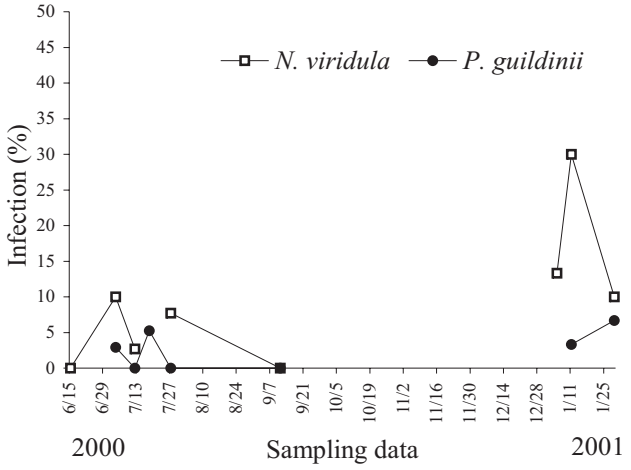


Figure 2. Trypanosomatid prevalence in stinkbugs collected in Londrina, north of Paraná State, during 2000-2001.

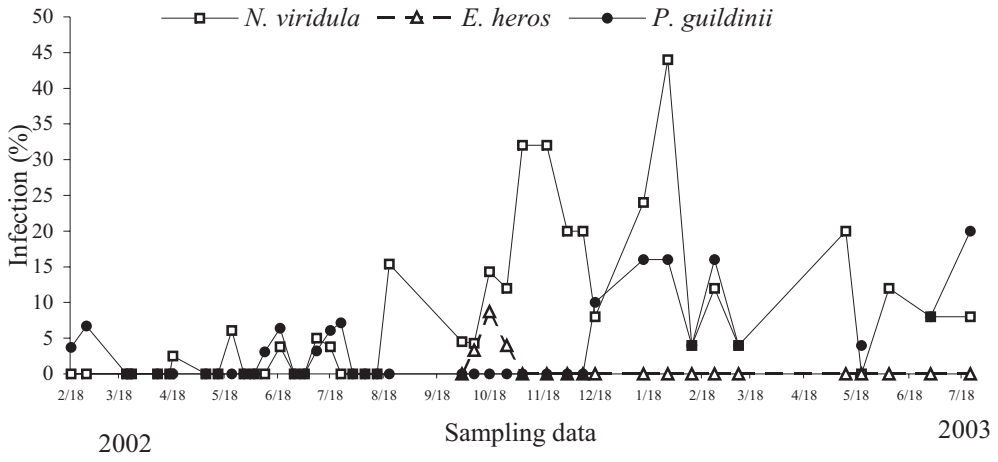


Figure 3. Prevalence of flagellates on stink bugs populations from Londrina, north of Paraná State, during 2002-2003.

we verified that the Florida flagellates reproduced very profusely in *Trichoplusia ni* cell cultures (TN368) immersed in TC100 medium (Life Technologies, Inc.), but *Spodoptera frugiperda* cells (Sf9) cultures were not permissive for the same trypanosomatids (D.R. Sosa-Gómez & D.G. Boucias, pers. observ.).

In the *N. viridula* samples from Quincy, FL, and in *P. guildinii* from Londrina, we found cysts attached to the flagellum (Fig. 1c). Cysts are thought to be forms of survival and propagation of trypanosomatids, and they occur in the *Blastocrithidia* and *Leptomonas* genera (Takata *et al*. 1996).

Although the mean size of body and flagella in the current study does not match those of Fuxa *et al*. (2000), there was an overlapping of the body and flagellum length. We observed a prevalence lower than that reported by Fuxa *et al*. (2000) in *N. viridula*, which reached almost 95% of the stink bugs infected. The higher number observed by Fuxa *et al*. (2000) could be due to the sampling method, because we sampled flagellates in the hemolymph, whereas these authors diagnosed infection by dissecting the alimentary tract of each individual. The infection peaks from November until March, were observed on soybean. At present little is known about the interactions between host plant species and levels of infection.

Unclean colonies of *N. viridula* in (food contaminated with feces) mass rearing had a high prevalence of infection (D.R. Sosa-Gómez & D.G. Boucias, unpub.), possibly due to the transmission of this pathogen through the feces left on the feeding substrate. The condensation of water on soybean plants early in morning may help the locomotion of the active phase and favor transmission. This behavior could explain the high prevalence during wet weather.

The presence of flagellates in *N. viridula* hemolymph is an indicative that flagellates can transpose the gut barrier more easily that in *E. heros*. This could be due to differences in the gut wall or in the flagellates virulence. Differences in virulence among trypanosomatid isolates have been observed by Baccan *et al*. (2001); for example, *Leptomonas* isolated from *E. heros* caused mortality very rapidly (24h after infection) in *Veneza zonata* (Pallas) but other isolates did not. Despite the high densities of flagellates in *N. viridula* and *P. guildinii* blood droplets, the insects were alive, revealing the nature of chronic diseases against these species. Until present the effect on longevity, fertility and fecundity is unknown.

Flagellates apparently do not invade and freely circulate in the hemolymph of *E. heros* as they do in *N. viridula* and *P. guildinii*. Their detection in *E. heros* required strong pressure on the body during the bleeding procedure. Thus, it is possible that they were released into the hemocoel after rupture of the gut wall. A *Leptomonas* sp. was detected in the digestive tract of *E. heros* by Batistoti (1998).

Low levels of trypanosomatid parasites in *E. heros* compared to *N. viridula* implies a lower susceptibility of this species. The mechanisms involved in these differences of susceptibility are unknown. Baccan *et al*. (2001) suggest that there are several mechanisms involved in the penetration of the gut wall towards the hemocoel, some of them rendering the wall permeable to the bacteria inhabiting the digestive tract.

The importance of pathogens as regulatory agents of insect populations is becoming more evident in several host-pathogen systems. Previously, life table studies of stink bugs usually did not consider pathogens as death cause. In biology studies stink bugs present high mortality rates but nothing is known about their etiological causes (Panizzi 1987). However pathogens such as viruses have been mentioned as the cause of mortality and diminished reproductive capability in the southern green stink bug (Williamson & Wechmar 1995). Flagellates could be a causal agent partly responsible for high mortality rates and reduced life span. Additional studies are necessary, because the impact of infection by these protozoans on pentatomid biology and reproduction at population level is still unknown. Although, trypanosomatid prevalence was higher than that observed for entomopathogenic fungi, they seem to be inefficient control agents in short term condition.

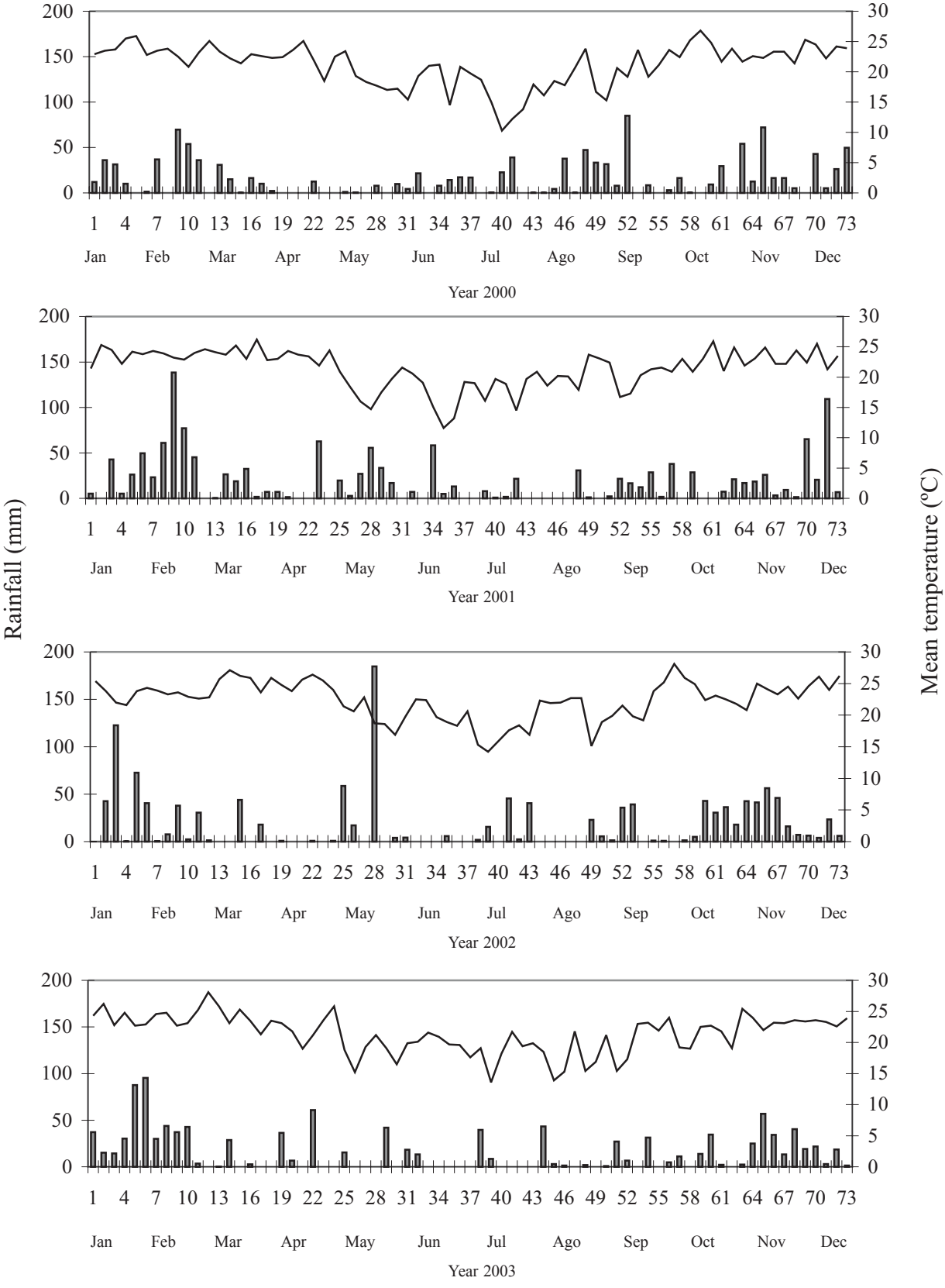


Figure 4. Rainfall (mm), mean temperature (°C) during 2000 to 2003. Number of days per year divided by five-day periods.

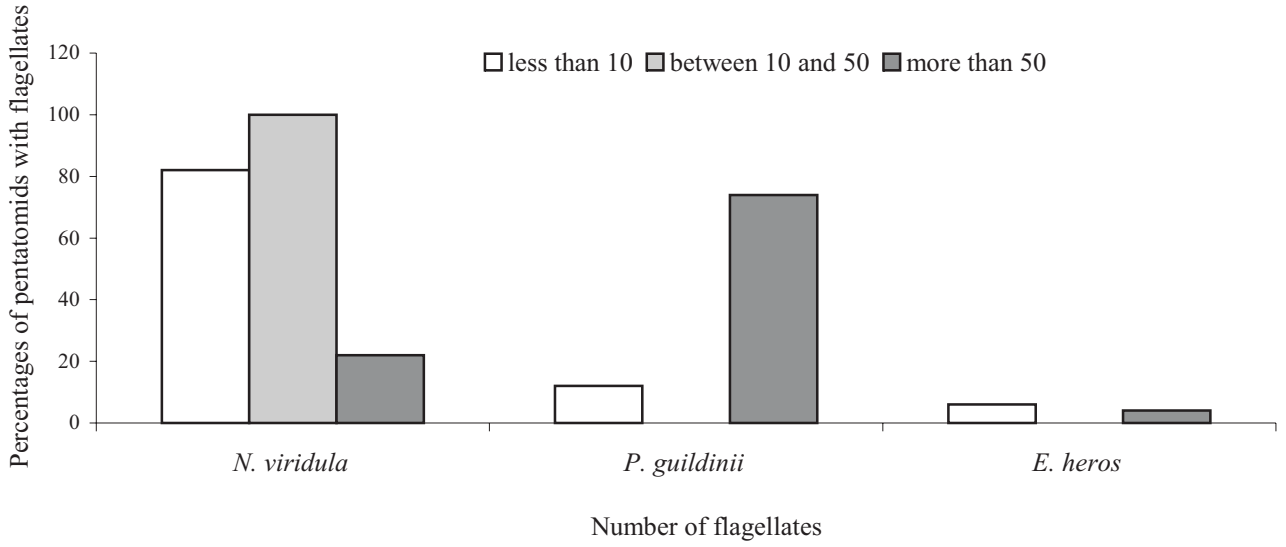


Figure 5. Frequency of flagellates in the hemolymph of pentatomids (*N. viridula, P. guildinii, E. heros*).

Acknowledgments

We thank Dr. James R. Fuxa for the critical review of the manuscript, and we acknowledge Jovenil José da Silva and José Jairo da Silva for their technical assistance. This work received financial support from the Embrapa Soja and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)**.** This paper was approved for publication by the editorial board of Embrapa Soja as manuscript 02/2004.

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Received 20/VI/04. Accepted 25/X/04.