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Infections of *Hypostomus* spp. by *Trypanosoma* spp. and leeches: a study of hematology and record of these hirudineans as potential vectors of these hemoflagellates

Infecções de *Hypostomus* spp. por *Trypanosoma* spp. e Sanguessugas: um estudo hematológico e registro desses hirudíneos como potenciais vetores desses hemoflagelados

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Abstract

Among Kinetoplastida, the *Trypanosoma* is the genus with the highest occurrence infecting populations of marine fish and freshwater in the world, with high levels of prevalence, causing influences fish health and consequent economic losses, mainly for fish populations in situation stress. This study investigated infections of *Hypostomus* spp. by *Trypanosoma* spp. and leeches, as well as blood parameters of this host in the network of tributaries of the Tapajós River in the state of Pará, in the eastern Amazon region in Brazil. Of the 47 hosts examined, 89.4% were parasitized by *Trypanosoma* spp. and 55.4% also had leeches attached around the mouth. The intensity of *Trypanosoma* spp. increased with the size of the host, but the body conditions were not influenced by the parasitism. The number of red blood cells, and hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), total number of leukocytes and thrombocytes showed variations and negative correlation with the intensity of *Trypanosoma* spp. in the blood of the hosts. The results suggest that the leeches were vectors of *Trypanosoma* spp. in *Hypostomus* spp.

Keywords: Amazon, body condition, hemoparasites, blood, *Trypanosoma* spp., *Hypostomus* spp.

Resumo

Dentre os Kinetoplastida, *Trypanosoma* é o gênero com maior ocorrência, infectando populações de peixes marinhos e de água doce em todo o mundo. Apresenta elevados níveis de prevalência, ocasiona impactos na saúde dos peixes e consequente perdas econômicas, principalmente para populações de peixes em situação de estresse. Este estudo investigou a infecção por *Trypanosoma* spp. e sanguessugas em *Hypostomus* spp. e parâmetros sanguíneos desse hospedeiro do sistema de tributários do Rio Tapajós, no Estado do Pará, Amazônia Oriental, Brasil. De 47 hospedeiros examinados, 89,4% estavam parasitados por *Trypanosoma* spp., e 55,4% tinham também sanguessugas na região da boca. A intensidade de *Trypanosoma* spp. aumentou com o tamanho dos hospedeiros, mas as condições corporais não foram influenciadas pelo parasitismo. O número de eritrócitos, hematócrito, hemoglobina, VCM, HCM, CHCM, número de leucócitos e trombócitos totais apresentaram variações e correlação negativa com a intensidade de *Trypanosoma* spp. no sangue dos hospedeiros. Os resultados sugerem que sanguessugas foram os vetores de *Trypanosoma* spp. in *Hypostomus* spp.

Palavras-chave: Amazônia, condição corporal, hemoparasito, sangue, *Trypanosoma* spp., *Hypostomus* spp.

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Introduction

The genus *Hypostomus* Lacepède, 1803 comprises small and large Loricariidae with a highly variable pattern of coloration, with or without spots. The abdomen may or may not be covered with scales; the caudal fin is forked, with the larger lobe on top; there are two or three pre-dorsal scales; five rows of scales on the caudal peduncle; and a caudal keel with or without lateral scales (ARMBRUSTER, 2004). However, there is still no consensus about the taxonomy of Loricariidae, indicating the need for more specific studies aimed at its accurate identification (ZAWADZKI et al., 2012).

Trypanosomatidae Doflein, 1901 (Kinetoplastida) species have a single nucleus, are elongated with a single flagellum or rounded with a very short flagellum, and are not free living. Many members of this family are heteroxenous, living one phase of life in the bloodstream or in a variety of tissues of different species of aquatic vertebrates (fish, amphibians and reptiles), and another phase in the intestine of bloodsucking invertebrates. *Trypanosoma* species (Gruby, 1843) are almost all heteroxenous and parasites of the blood of all classes of vertebrates, including marine and freshwater teleost and elasmobranch fish all over the world (WOO, 1998; EIRAS et al., 2008; ROBERTS & JANOVY, 2013; HAYES et al., 2014). Today, more than 200 species are known to parasite fish around the world (GUPTA & GUPTA, 2012). More than 60 of these *Trypanosoma* species have been recorded in fish in Brazilian hydrographic basins and approximately 18 species (almost one third) have been described in Loricariidae species (EIRAS et al., 2010).

Trypanosoma species are transmitted to fish through a blood-sucking host, usually a species of leech. However, isopod crustaceans can also be potential vectors of trypanosomiasis in fish. These hemoparasites may not be detrimental to the infected fish, but in some cases can cause severe alterations of blood. Some trypanosomatids are highly pathogenic and can cause the death of the host fish (ISLAM & WOO, 1991; WOO, 1998; AHMED et al., 2011; LEMOS et al., 2015; MAQBOOL & AHMED, 2016). However, the effects of these hemoflagellates on the host fish physiology are not well understood. In general, it is difficult to determine unequivocally, based solely on morphology, when a *Trypanosoma* species is undescribed. DNA sequence data can provide some insight into species designations (WOO, 1998; LEMOS et al., 2015), but there are few studies for these hemoflagellates in South America. Therefore, knowledge of these parasites hematozoa Neotropical fish remains limited.

The purpose of this study was to investigate infection by *Trypanosoma* spp. and leeches in *Hypostomus* spp., and the hematology of these hosts in the Tapajós River system in the state of Pará, Brazil.

Materials and Methods

Fish and collection site

Between September and October 2012, 47 species of *Hypostomus* spp. were collected, 17 from the Uruá Stream (S 0,4°31'58,7", W 56°18'2,2") and 30 from the mouth of the Jamaxinzinho River (S 0,4°53'58,0", W 56°27'00,3"), which are tributaries of the Tapajós River system in the state of Pará, in northern Brazil. The fish were collected during an inventory of the region's ichthyic and parasitic fauna to determine the components of the diversity of this biome prior to the construction of a complex of hydroelectric plants, in order to garner statistical data to underpin the assessment, prediction and mitigation of the consequences of the anthropogenic changes imposed on the fish in the rivers that run through these protected areas (Figure 1).

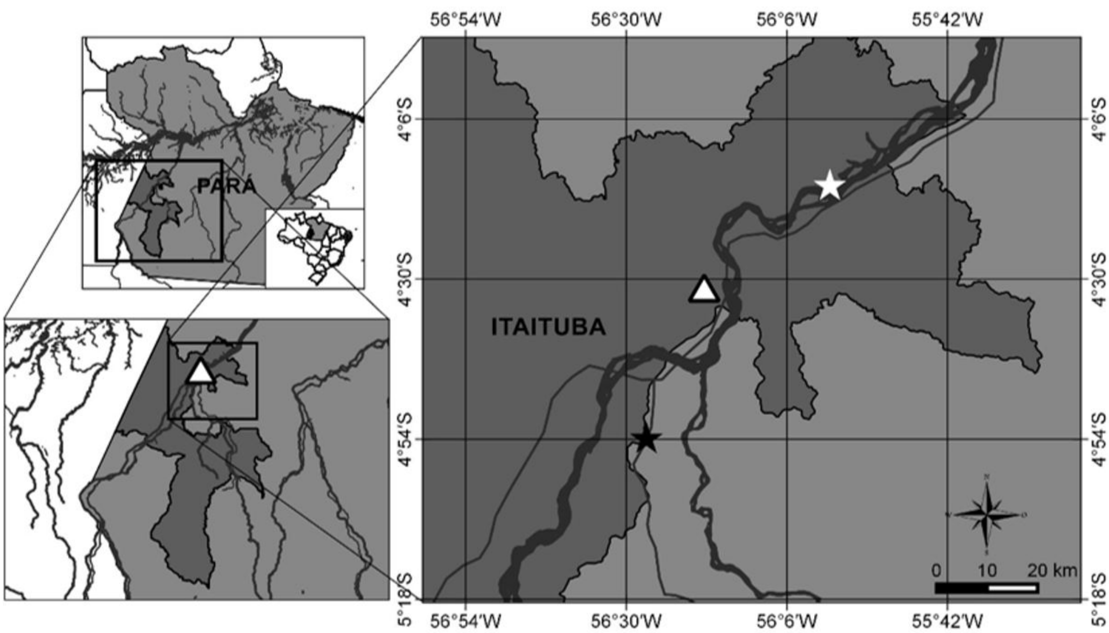

Figure 1. Geographic location of collection points. Town of Itaituba Uruá Stream (S 0.4°31'58.7", W 56°18'2.2"); Jamaxinzinho river (S 0.4°53'58.0", W 56°27'00.3"), Pará, Brazil.

Collection and analysis of ectoparasites and hemoparasites

Leeches were collected from the oral region (Figure 2) of the specimens of *Hypostomus* spp., and then fixed in alcohol. To examine the hemoparasites, a blood sample was collected by cardiac puncture using 1.5 mL syringes containing sodium heparin (25,000 Ul/mL). The blood samples were collected at the collection site. Part of the blood (8 μL) was used to prepare blood smears with May-Grünwald-Giemsa stain (DACIE & LEWIS, 2007). These blood smears were used to quantify the *Trypanosoma* spp. in each host. The ecological terms used here were those recommended by Rohde et al. (1995) and Bush et al. (1997).

Extensions containing the blood parasites and leeches were deposited in the collection of the Continental Fish Hematology Laboratory – CEPTA/ICMBio in Pirassununga, state of São Paulo. All the *Hypostomus* sp. specimens were deposited in the fish collection of the Genetics Museum of UNESP at Botucatu, state of São Paulo.

Blood collection and analysis procedures

The remaining blood was used to determine the total number of erythrocytes and the hemoglobin and hematocrit concentration. This data was then used to calculate the mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). The blood smears were also used to determine the total white blood cells and thrombocytes (DACIE & LEWIS, 2007).

The body weight (g) and total length (cm) data were used to calculate the relative condition factor (Kn) of the host fish, which was then compared with the standard value (Kn = 1.00) by means of the Mann-Whitney (*U*) test. The Spearman correlation coefficient (*rs*) was used to verify possible correlations between the intensity of *Trypanosoma* spp. and the weight, length and blood parameters of the hosts (ZAR, 2010). In addition, weight and length data were used to calculate the length-to-weight ratio ($W = aL^b$) after logarithmic transformation of length and weight and subsequent two straight-line adjustments, thereby obtaining lny = lnA + Blnx (LE-CREN, 1951).

Results

The fish weighed 162.3 ± 95.6 g and were 25.1 ± 7.5 cm long. The pH of the Uruá Stream was 7.4 and its water temperature was 27.0 °C, while the pH at the mouth of the Jamaxinzinho River was 7.0 and its water temperature was 29.9 °C.

Were examined 47 fish of which 42 were infected with *Trypanosoma* spp. with their distribution in the area of collecting and presenting different types of *Trypanosoma* spp. (Table 1).

The shape of the *Trypanosoma* spp. was long and wide, with tapered ends and a highly prominent undulating membrane, with several folds. The kinetoplast was terminal or sub-terminal, round and somewhat stained, surrounded by a clear area. The nucleus,

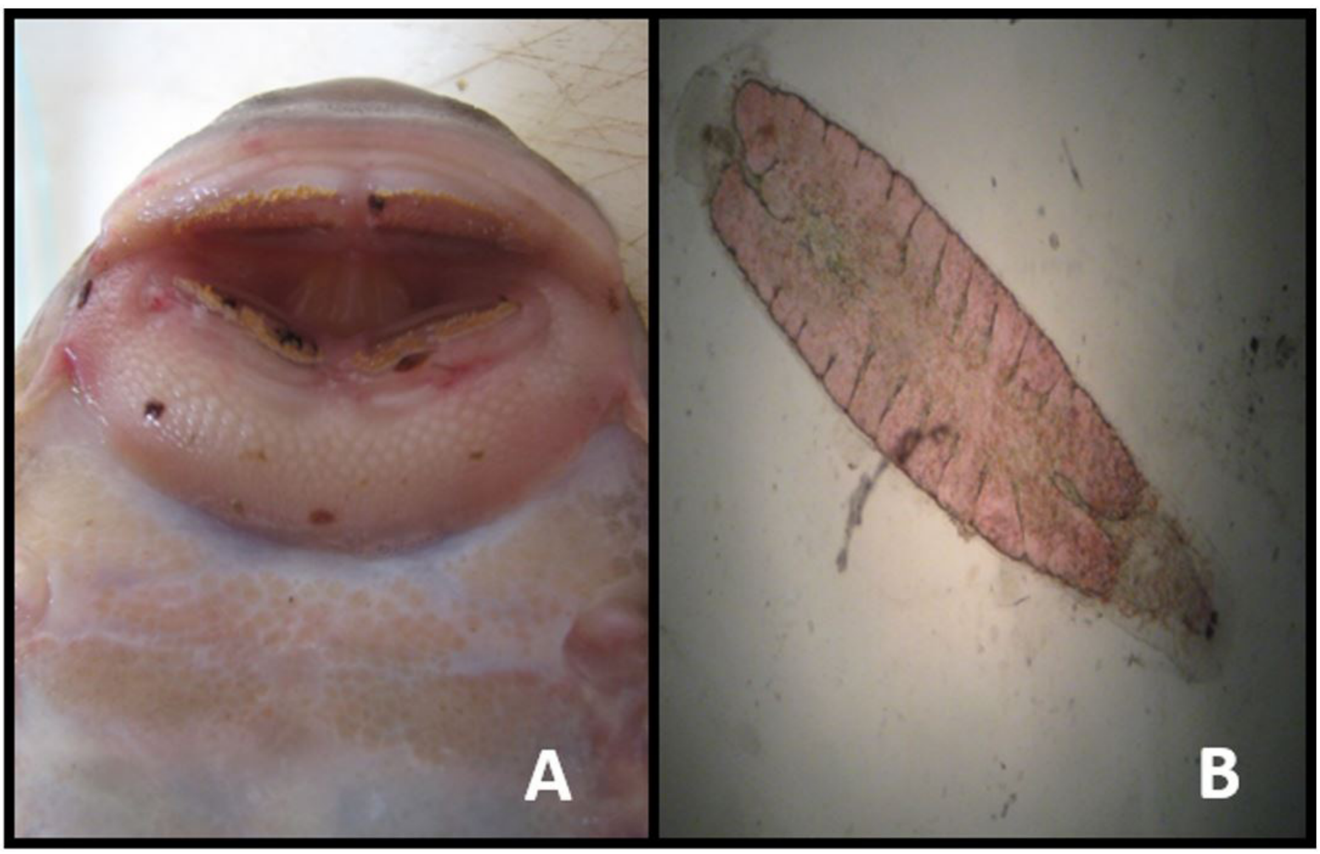

Figure 2. (A) Leeches in the oral region of *Hypostomus* sp.; (B) leech, image of leech in light microscope (10 × magnification).

rounded to oval in shape and slightly stained, was located centrally and occupied the entire width of the cell, with few or no granulations. Its cytoplasm was highly granular and it had numerous vacuoles along the body. The flagellum was short, slightly stained and, in some cases, almost undetectable (Figure 3).

Apparently, one sees three distinct morphological types of *Trypanosoma* spp. Thus, we present a description of trypomastigotes in fish examined, highlighting the polymorphism with their respective measures (Table 2).

All the *Hypostomus* spp. specimens parasitized by leeches also presented *Trypanosoma* spp. infection. There was variation in the intensity of *Trypanosoma* spp. and leeches in the hosts examined (Table 3). The intensity of *Trypanosoma* spp. in the blood was positively correlated with the length (rs = 0.622, p = 0.0001) and weight (rs = 0.426, p = 0.003) of the hosts.

The equation for the weight-length relationship of *Hypostomus* spp. revealed a negative allometric relationship (Figure 4), indicating that there was a greater increase in body weight than in length. The Kn of hosts varied (Table 4), but did not differ (U = 940.0, p = 0.213) from the standard value (Kn = 1.00).

The intensity of *Trypanosoma* spp. in the blood correlated negatively with the hematocrit (rs = –0.796, p = 0.0001), MCV (rs = –0.731, p = 0.0001), MCH (rs = –0,555, p = 0.0001), MCHC (rs = –0,777, p = 0.0001) and the total number of leukocytes (rs = –0.352, p = 0.018) of the hosts. However, a positive correlation was found between the intensity of *Trypanosoma* spp. and hemoglobin (rs = 0.435, p = 0.003) and the total number of red blood cells (rs = 0.640, p = 0.0001).

Table 1. Morphotypes *Trypanosoma* number of infected fish and collection sites along the Tapajos River.

Trypanosoma	**Infected fish**	**Collection site**
Morphotype I	8	Uruá Stream
Morphotype II	7	Uruá Stream
Morphotype III	27	Jamaxinzinho River

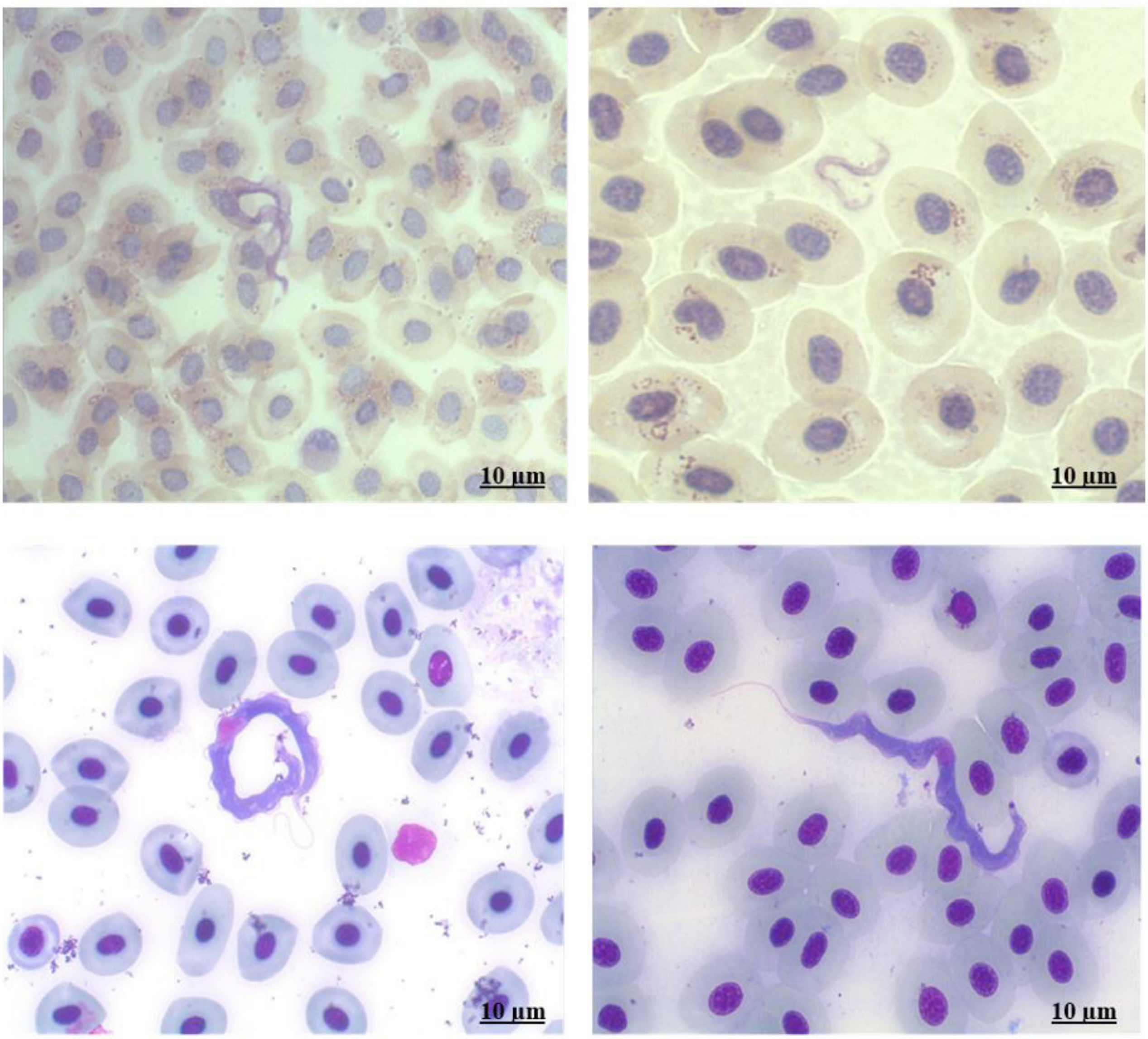

Figure 3. Forms of trypomastigotes viewed in light microscope. The blood smears of *Hypostomus* spp. were stained with Grünwald-Giemsa.

Table 2. Measures of morphometric characteristics with the values mean (minimum and maximum) expressed in µm of blood forms of *Trypanosoma* spp.

	Morphotype I (n = 5)	Morphotype II (n = 4)	Morphotype III (n = 8)
FL	18.0 (7.1-34.8)	16.7 (9.0-21.3)	23.0 (20.0-33.0)
UM	1.0 (0.6-2.5)	1.1 (0.6-1.4)	1.8 (1.2-2.5)
BL	50.4 (21.9-87.1)	49.3 (28.4-72.9)	55.1 (34.1-75.5)
BW	2.1 (0.6-4.2)	2.1 (0.6-3.6)	2.4 (2.0-2.8)
NL	4.9 (2.0-12.1)	4.8 (2.9-6.5)	4.2 (2.5-5.6)
NW	2.0 (0.6-4.0)	1.9 (0.6-3.6)	1.6 (1.3-2.5)
PD	25.1 (7.1-50.1)	26.0 (14.8-43.9)	27.5 (12.5-43.1)
FA	23.2 (8.4-39.8)	23.2 (12.3-30.3)	22.1 (16.9-28.0)
MK	20.0 (6.4-32.0)	21.9 (12.3-27.3)	20.1 (14.6-29.2)
PK	1.4 (0-5.2)	1.5 (0-5.4)	2.0 (1.2-4.8)

n: number trypanosomes measured; FL: flagellum length; UM: Width of the undulating membrane; BL: body length; BW: body width; NL: nucleus length; NW: nucleus width; PD: distance from the front end of the nucleus; FA: distance from the posterior end nucleus; MK: midnucleus to kinetoplast and PK: posterior to kinetoplast.

Table 3. Parasitological indices in *Hypostomus* spp. from Tapajós river system, state of Pará (Brazil). Count of *Trypanosoma* spp. in 8 µl of per host.

Parameters	Hirudinea	*Trypanosoma* spp.
Examined fish	47	47
Parasitized fish	26	42
Prevalence (%)	55.3	89.4
Mean intensity	3.1	11.6
Mean abundance	1.7	10.8
Range of intensity	1-7	2-35
Total number of parasites	80	487

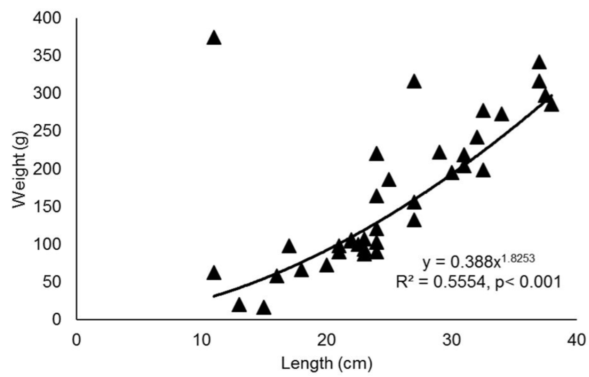

Figure 4. Weight-length relationship for *Hypostomus* spp. from Tapajós river system, Pará state (Brazil) parasitized by *Trypanosoma* sp. and leeches.

Table 4. Hematological parameters of *Hypostomus* spp. (N = 47) from Tapajós system river, state of Pará (Brazil) parasitized by *Trypanosoma* spp. and leeches.

Parameters	Mean ± SD	Minimum-Maximum
Kn	1.00 ± 0.14	0.69-1.73
Red blood cells ($x10^6/\mu L$)	0.455 ± 0.184	0.157-0.691
Hematocrit (%)	21.4 ± 9.1	8.0-37.0
Hemoglobin (g/dL)	2.3 ± 0.1	2.2-2.4
MCV (fL)	683.3 ± 576.0	118.9-2038.2
MCH (pg)	62.6 ± 32.2	33.4-144.9
MCHC (g/dL)	13.2 ± 6.4	6.0-29.9
White blood cells (µL)	28,840 ± 14,535	9090-54,600
Thrombocytes (µL)	27,861 ± 11,088	8700-56,840

MCV: Mean corpuscular volume; MCHC: Mean corpuscular hemoglobin concentration; MCH: Mean corpuscular hemoglobin.

Discussion

Three morphotypes of *Trypanosoma* were found in *Hypostomus* spp., being two in hosts from Uruá Stream and one in hosts from Jamaxinzinho River. In addition, a high level of parasitism of *Trypanosoma* spp. and leeches in *Hypostomus* species from Tapajós River system was found. Fujimoto et al. (2013) reported low prevalence and high intensity of *Trypanosoma* spp. and leeches in *Hypostomus* species from the Guamá River. In marine and freshwater fish populations, *Trypanosoma* spp. maintain their life cycle by using hematophagous invertebrates as primary hosts, namely leech species, and subsequently fish populations (D'AGOSTO & SERRA-FREIRE, 1993; WOO, 1998; PÁDUA et al., 2011; HAYES et al., 2014; LEMOS et al., 2015). *Trypanosoma* spp. multiply in the digestive tract of leeches, with the non-infective forms migrating to the proboscis, where they transform into infective forms and are inoculated into fish when the leech feeds (WOO, 1998). In Brazil, it has been suggested that the main vector of these hemoflagellates in fish from the Loricariidae family is the *Batracobdella gemmata* leech (D'AGOSTO & SERRA-FREIRE, 1993). Therefore, in *Hypostomus* spp., the transmission to these hosts presumably occurs infected leeches feed on them. Moreover, the wide diversity of the leech fauna of the Neotropical region (SKET & TRONTELJ, 2008) leads to the assumption that the hemoflagellate fauna of this region is also diverse.

Species of *Trypanosoma* can cause anorexia in infected fish. This is most evident in cases of high parasitemia, although fish that survive the disease return to normal feeding. Anemia may alter the hosts' body conditions and the somatic indices of their liver, spleen and heart (WOO, 1998). However, the hosts' body conditions in this study were not affected by parasitism, as indicated by the relative condition factor. In addition, stress conditions may influence the course of parasitemia in fish. At low and high temperatures, fish have decreased trypanosome levels in the blood (WOO, 1998; GUPTA & GUPTA, 2012).

Trypanosomes can cause anemia in infected fish (GUPTA & GUPTA, 2012; MAQBOOL & AHMED, 2016), whose erythrocytes frequently undergo alterations. As trypanosomes generally depend on the energy resources of the host fish, the impact of this parasitism in fish is considerable, ranging from physiological, metabolic, pathological and biochemical alterations to asymptomatic behavior. Woo (1998) stated that anemia caused by *Trypanosoma* spp. may be related to the inactivity of the host's hemopoietic system. The severity of this anemic process is directly tied to the intensity of hemoparasite load, and is partly caused by lytic factors and hemodilution. The lytic factor is secreted by living parasites and lyses red blood cells (RBC) in the absence of specific antibodies. It seems that the virulence factor that leads to anemia is a protease. However, Fujimoto et al. (2013) reported that the RBC parameters of *Hypostomus* sp., *Ancistrus* sp. and *Rineloricaria lanceolata* were not influenced by infection by *Trypanosoma* sp., but the RBC and hematocrit level in *Lasiancistrus saetiger* increased while hemoglobin concentration decreased. The RBC count, hematocrit and MCV of *Hypostomus* spp. in this study were similar to those reported by Fujimoto et al. (2013) for the same host infected by *Trypanosoma* spp., while the hemoglobin concentration, MCHC and MCH levels were lower. The hemoglobin concentration and RBC number showed an increase with the intensity of *Trypanosoma* spp. in blood. However, the hematocrit, MCV, MCH and MCHC of the hosts of this study presented negative correlation with the intensity of *Trypanosoma* spp. in blood, indicating that an increase in parasite number may lead fish to an anemiant process.

Hemostasis is a function of paramount importance when fish are responding to injuries, and piscine thrombocytes play a central role in this process. The number of thrombocytes can vary from 2,000-78,900 µL among healthy fish species due to intraspecific variations, which are attributed to biotic factors such as age, season and maturity, and abiotic factors such as water temperature, pH, dissolved oxygen content, sex, and maturity stage, as well as stress and diseases (TAVARES-DIAS & OLIVEIRA, 2009). However, the number of thrombocytes in *Hypostomus* spp. suggests that it was not influenced by the infection of *Trypanosoma* spp., since the host's hemopoietic system was not impaired.

Piscine leukocytes are involved in phagocytosis, immunoglobulin production, modulation of immune defense, inflammation process and defense against parasitic and bacterial infections and stress (DAVIS et al., 2008; RANZANI-PAIVA et al., 2013). Leukocytosis has been reported in *Schizothorax plagiostomus* infected with *Trypanosoma* spp. (MAQBOOL & AHMED, 2016), while lymphocytes decreased perceptibly in *Hypostomus* sp. (FUJIMOTO et al., 2013). In contrast, the total number of leukocytes in *Hypostomus* sp. appeared to have been unaffected by *Trypanosoma* spp. infection.

In summary, armored catfish species are usually infected by *Trypanosoma* spp., particularly the *Hypostomus* species. *Hypostomus* spp. sampling was carried out in dry season, when the water levels in lakes and streams decrease drastically, resulting in greater competition for resources such as food and shelter. Therefore, these factors combined with high temperatures may be related to the high infection levels that were found, representing a stress condition that reduces the immune status of the fish population and thus facilitating the survival of parasites. The morphometric features, alone, did not suffice to identify the species of *Trypanosoma* found. Thus, a review of the *Trypanosoma* species that infect fish species is needed, along with other factors such as host characteristics, isolation culture media, experimental infections and analysis of DNA sequences. Finally, this is first study of hematological parameters and infection by *Trypanosoma* in *Hypostomus* spp. parasitized by hirudineans in the Tapajós River system.

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