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Cytometric and morphological characterization of hemocytes from four species of freshwater decapod crustaceans

Caracterización citométrica y morfológica de los hemocitos de cuatro especies de crustáceos decápodos de agua dulce

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ABSTRACT. Characterization and classification of invertebrate hemocytes is a powerful tool for determining the physiological effect of environmental stress. Hemocytes from four decapod crustacean species *Dilocarcinus pagei*, *Zilchiopsis collastinensis*, *Aegla uruguayana* and *Macrobrachium borellii* were analyzed by flow cytometry followed by morphological characterization by optical microscopy. Based on morphological cell characteristics, three categories of hemocytes were identified: granulocytes with abundant granularity accounting for 1.87% (*D. pagei*), 5.23% (*Z. collastinensis*), 0.98% (*A. uruguayana*) and 2.41% (*M. borellii*), semigranulocytes with lower granularity accounting for 2.22% (*D. pagei*), 4.81% (*Z. collastinensis*), 2.38% (*A. uruguayana*) and 68.19% (*M. borellii*) and, finally, hyalinocytes with almost no granularity accounting for 89.87% (*D. pagei*), 65.75% (*Z. collastinensis*), 92.33% (*A. uruguayana*) and 22.04% (*M. borellii*). Analysis of the total hemocyte count (cells/ml) showed the following results: 4.3 ± 0.58 (x10^6) in *D. pagei*, 2.3 ± 0.33 (x10^6) in *Z. collastinensis*; 3.1 ± 0.56 (x10^6) in *A. uruguayana* and 2.6 ± 0.25 (x10^6) in *M. borellii*. This study demonstrates the applicability of flow cytometry to effectively differentiate and enumerate circulating hemocytes in native crustacean species, providing novel insights into the use of this method for assessing physiological status in these species. In addition, interspecific variability in these cellular parameters was observed. The results contribute to the understanding of the innate immune system in these four crustacean species and allow establishing a baseline to identify welfare or stress conditions in these animals.

Keywords: Crustacea, flow cytometry, hemolymph, hemocyte analysis.

RESUMEN. La caracterización y clasificación de los hemocitos de invertebrados es una poderosa herramienta para determinar el efecto fisiológico del estrés ambiental. Los hemocitos de cuatro especies de crustáceos decápodos *Dilocarcinus pagei*, *Zilchiopsis collastinensis*, *Aegla uruguayana* y *Macrobrachium borellii* se analizaron mediante citometría de flujo seguida de una caracterización morfológica por microscopía óptica. Basándose en las características morfológicas celulares, se identificaron tres categorías de hemocitos: granulocitos con granularidad abundante que representaban el 1,87% (*D. pagei*), el 5,23% (*Z. collastinensis*), el 0,98% (*A. uruguayana*) y el 2,41% (*M. borellii*), semigranulocitos con menor granularidad con un 2,22% (*D. pagei*), 4,81% (*Z. collastinensis*),

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2,38% (*A. uruguayana*) y 68,19% (*M. borellii*) y, por último, hialinocitos casi sin granularidad con un 89,87% (*D. pagei*), 65,75% (*Z. collastinensis*), 92,33% (*A. uruguayana*) y 22,04% (*M. borellii*). El análisis del recuento total de hemocitos (células/ml) arrojó los siguientes resultados: 4,3 ± 0,58 (x10^6) en *D. pagei*, 2,3 ± 0,33 (x10^6) en *Z. collastinensis*; 3,1 ± 0,57 (x10^6) en *A. uruguayana* y 2,6 ± 0,25 (x10^6) en *M. borellii*. Este estudio demostró que la citometría de flujo es eficaz para diferenciar y contar los hemocitos circulantes, lo que la convierte en una herramienta valiosa para evaluar el estado fisiológico. Además, se observó variabilidad interespecífica en estos parámetros celulares. Los resultados contribuyen a la comprensión del sistema inmunitario innato en estas cuatro especies de crustáceos y permiten crear una línea de base para identificar condiciones de bienestar o estrés en estos animales.

Palabras clave: Crustáceos, citometría de flujo, hemolinfa, análisis de hemocitos.

INTRODUCTION

Crustaceans represent one of the most diverse animal groups globally, exhibiting various appearances and lifestyles (Söderhäll & Söderhäll, 2022). In Argentina, freshwater decapod crustaceans constitute the macroinvertebrates with the highest biomass, playing significant roles in community dynamics (Collins *et al*., 2012). They occupy an intermediate position in food webs and facilitate the transfer of matter and energy between aquatic and terrestrial environments (Williner, 2010; Carvalho *et al*., 2018). This group displays a broad geographic distribution (Morrone & Lopretto, 1994; de Melo, 2003) inhabiting various aquatic environments (Collins *et al*., 2007). Consequently, their habitats are diverse, with different taxa establishing unique relationships with their surroundings.

Crustaceans possess an open circulatory system in which hemolymph flows through the hemocoel, with its components and hemocytes playing key roles in tissue nourishment and defense mechanisms, including coagulation, wound repair, and immune responses. Hemocytes are generated throughout the animal's life in two types of tissues: the hematopoietic tissue (HPT) and the anterior proliferation center (APC) (Jiravanichpaisal *et al*., 2006; Lin *et al*., 2011; Kruangkum *et al*., 2025).

Hemocytes in crustaceans are typically classified by morphology, cytochemistry, and function (Hose *et al*., 1990). Generally, morphological classification distinguishes three cell types (Bauchau, 1980). The main types include hyalinocytes (H), which are small and non-refractile; granulocytes (G), which have abundant cytoplasmic granules; and semigranulocytes (SG), with numerous small granules (Aguirre-Guzmán *et al*., 2009; Söderhäll, 2016). Functionally, H and SG perform phagocytosis, while encapsulation involves SG and G. Granulocytes also release components essential for melanization through degranulation and the activation of the prophenoloxidase system (Cerenius & Söderhäll, 2012).

Carus (1824) made the first characterizations of hemocytes in crustaceans in the early XIX century. Despite efforts to create a unified nomenclature, such as those by Bauchau (1980) and Hose (1987, 1990), controversies persist regarding classification methods and criteria. For instance, Battison *et al*. (2003) identified eleven hemocyte types in the American lobster (*Homarus americanus*), while Sung and Sun (2002) recognized six subtypes in *Penaeus monodon* using monoclonal antibodies, contrasting with three types identified morphologically. Similarly, Lv *et al*. (2014) characterized three hemocyte types in the Chinese mitten crab (*Eriocheir sinensis*), and dos Santos *et al*. (2023) identified three types in the tropical marine amphipod *Parhyale hawaiensis*. This inconsistency complicates comparisons across species. Zhang *et al*. (2006) identified

three hemocyte types in the Chinese shrimp (*Fenneropenaeus chinensis*) and proposed two hemocyte lineages. The variation in terminology hampers the comparison of blood cells among different crustacean species. Nevertheless, the most widely accepted morphological classification is that of Bauchau *et al*. (1980), revised by Söderhäll (2016). Understanding these classifications is essential for identifying species-specific indicators of environmental health.

The identification and classification of crustacean hemocytes are essential to understanding their immune roles and facilitating comparisons across species (Gargioni & Barracco, 1998). Knowledge of hemocytes also aids in assessing the impacts of environmental stress on crustaceans (Lv *et al*., 2014). Flow cytometry (FC) has largely replaced microscopic evaluation, offering rapid cell type discrimination by size and complexity (Shapiro, 2003). This technique enables the processing of more samples, analysis of more hemocytes per sample, and enhances statistical reliability (Oliver *et al*., 2011). Flow cytometry has been effectively used to examine hemocytes in various crustacean species (Jia *et al*., 2017).

Crustaceans exhibit cellular changes in response to various extrinsic and intrinsic factors, including environmental stress, molt cycles, and nutritional status (Söderhäll, 2016; Hong *et al*., 2017; Day *et al*., 2019; Qyli *et al*., 2020; Frizzera *et al*., 2022; Yifei *et al*., 2024). Total hemocyte count (THC) and differential hemocyte count (DHC) serve as stress indicators (Lorenzon *et al*., 2001) and stable immune parameters, making them valuable for monitoring crustacean health (Mix & Sparks, 1980; Battison *et al*., 2003). The success of defense responses relies on the quantity and types of hemocytes involved (Russo *et al*., 2001). Both THC and DHC are effective measures of an animal's physiological status. Characterizing hemocytes in natural conditions is crucial for understanding cellular responses to environmental, anthropogenic, and pathological stress (Donaghy *et al*., 2010).

There are no studies describing the classification of the hemocytes of *Dilocarcinus pagei* (Stimpson, 1861 - Trichodactylidae), *Zilchiopsis collastinensis* (Pretzmann, 1968 - Trichodactylidae), *Aegla uruguayana* (Schmitt, 1942 - Aeglidae) and *Macrobrachium borellii* (Nobili, 1896 - Palaemonidae), four species of decapod crustaceans that inhabit different water bodies of the Paraná River, Argentina. Our study aimed to identify and characterize the hemocytes of these species, belonging to three families with different evolutionary histories, and to establish a baseline for assessing the welfare status of these animals under normal conditions.

MATERIAL AND METHODS

Sampling and maintenance of specimens

Adults of *Aegla uruguayana* and *Macrobrachium borellii* were collected in September 2023 from 'El Espinillo' stream (31°47'09"S, 60°18'57"W), Entre Ríos province, Argentina. Adults of *Dilocarcinus pagei* and *Zilchiopsis collastinensis* were collected in July 2023 from 'Setúbal' shallow lake (31°35'27"S, 60°37'59"W), Santa Fe province, Argentina. These sampling sites showed low human impact, were located far from cultivated areas, and exhibited low concentrations of pesticides. The Espinillo stream is characterized by alternating rocky and sandy sections along its longitudinal course, with a predominance of allochthonous riparian vegetation. The flow varies between fast and slow, mainly influenced by rainfall. On the other hand, Setúbal shallow lake presents a predominantly sandy bed, abundant floating vegetation and a mainly slow water flow. Within the family Trichodactylidae, individuals of *D. pagei* live among aquatic vegetation, while organisms of *Z. collastinensis* live in burrows constructed of fine sediments. Within the family Aeglidae individuals of *A. uruguayana* live on the bottom using debris, rocks or tree trunks to hide. Finally, individuals of the species *M. borellii* (Palaemonidae) live all or part of their lives in the water column. A multiparameter sensor (Hatch HQ40D) was used to record water temperature, dissolved oxygen, pH and dissolved solids at both sites (Table 1).

Table 1. Environmental parameters
Tabla 1. Parámetros ambientales

	Sites	
Environmental parameters	"El Espinillo" stream	"Setúbal" lagoon
Conductivity (µs/cm)	1164	93.3
Temperature (ºC)	18.3	14.5
Dissolved oxygen (mg/l)	1.59	11.21
Oxygen saturation (%)	17	109
pH	7.5	8

Ten individuals of both sexes per species were used. Samples were transported alive to the laboratory, where they were acclimatized and processed within 24 hs after collection. Oxygen and shelter (rocks or artificial shelters) were provisioned to replicate natural conditions. The cephalothorax width of the crabs used was 35.22 ± 8.83 mm in *D. pagei* and 55.90 ± 5.17 mm in *Z. collastinensis*. The cephalothorax length of *A. uruguayana* was 22.09 ± 2.54 mm and of *M. borellii* was 25.94 ± 2.51 mm. In all cases, measurements were made with a digital caliper (± 0.01 mm).

Hemolymph extraction and cell analysis

Specimens were cryoanesthetized prior to hemolymph extraction. Hemolymph was extracted from the arthrodial membrane at the intersection between the cephalothorax and pereiopods (*A. uruguayana*, *D. pagei* and *Z. collastinensis*) and at the intersection of the abdomen and cephalothorax (*M. borellii*). From each individual, a 400 µL sample of hemolymph was collected using a sterile 1 mL syringe with a 29 gauge needle and previously rinsed with an anticoagulant solution of sodium citrate (10%). All practices were framed within the protocol proposed by Sneddon (2015) and approved by the experimental work ethics and safety committee (CEYSTE-CCT-Santa Fe, Argentina). The samples were placed in Eppendorf microtubes preloaded with the anticoagulant solution mentioned above in a 1:1 ratio (400 µl of hemolymph and 400 µL of anticoagulant). The samples were immediately centrifuged at 1000 g at 4 °C for 10 min (Sartorius A-14C). Cell pellets were suspended in 1X PBS for cytometric analysis and stored at 4 °C until cell determinations, which were performed on the same day of extraction.

Morphological and cytometric characterization

For morphological characterization, 10 µL of pure hemolymph, unstained and without anticoagulant, was immediately placed on a slide and spread under coverslip. The slides were then observed with an optical microscope (Nikon YS100) and a phase contrast microscope (Leica DM3000 LED) and the images were analyzed with LAS (V4.7) software to determine the size, shape and presence or absence of granules in the cytoplasm. In hemocytes, both the diameter for spherical cells and the major axis for cells with a slightly ellipsoidal shape were measured. Flow cytometric analysis was performed with GUAVA Easycyte – Millipore (5HT HT) under excitation by a 488 nm blue laser. Kaluza software (v2.2) (Beckman Coulter, 2023) was used to define population subsets and exclude unwanted events. At least 20000 cells were recorded for each sample. To visualize the data, a dot plot was used, in which each dot represents an event showing the corresponding value of each variable (size versus complexity). This plot of internal complexity (SSC: side scattered light) versus size (FSC: front scattered light) was used to discriminate between hyalinocytes, semigranulocytes and granulocytes according to their relative cell size and granularity and to estimate the percentage differences between them. The cell density of the four decapod crustacean species was also estimated to determine the total hemocyte count (THC).

Statistical analysis

Data analysis was performed by using RStudio (v4.3.2) software (2023). Data were checked for normal distribution (Shapiro-Wilk's test) and homogeneity of variances (Levene's test). Data were presented as the mean values ±

standard deviation (SD). Statistical significance was determined by one-way analysis of variance (one-way ANOVA) or through a non-parametric method (Kruskal-Wallis) according to normality and homogeneity of variances and then multiple comparison tests were performed in cases where statistical differences existed. Differences were considered significant at $p < 0.05$ (Zar, 1996).

RESULTS

Morphological characterization of hemocytes

Based on cell size and the abundance of intracellular granules, three main types of hemocytes were identified in the four species of decapod crustaceans: hyalinocytes (H), semigranulocytes (SG), and granulocytes (G). Generally, the shape of the three types of hemocytes was round. Analysis of the microscopy images showed an increase in granularity from hyaline to granular cells. A reduction in size from granular to hyaline hemocytes was observed (Figure 1a,c). Granules with refringence were observed in the SG and G of *A. uruguayana* (Figure 1c) and *M. borellii* (Figure 1d) whereas more defined hemocytes were observed in *D. pagei* (Figure 1a) and *Z. collastinensis* (Figure 1b). Table 2 presents the size of the three cell types in the four crustacean species analyzed.

Statistical analyses revealed significant differences ($p < 0.05$) in the size of each hemocyte type among the four decapod crustacean species (Figure 2). *Dilocarcinus pagei* and *Z. collastinensis* showed significant differences in H size, and both species also differed significantly from *A. uruguayana* and *M. borellii* (Figure 2a). Among the species, *Z. collastinensis* exhibited

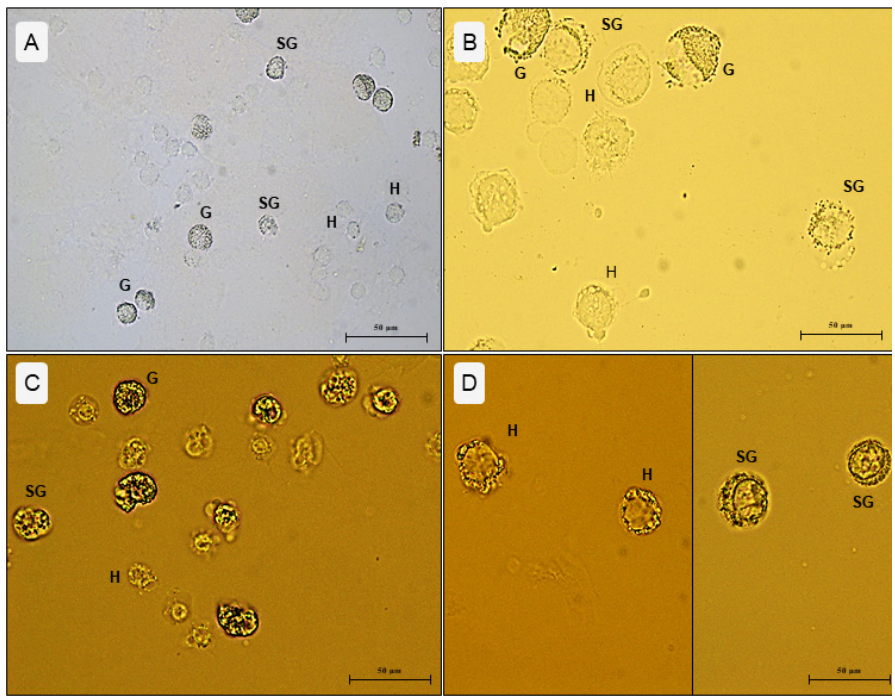

Figure 1. Hemocytes of A. *Dilocarcinus pagei*, B. *Zilchiopsis collastinensis*, C. *Aegla uruguayana* and D. *Macrobrachium borellii* observed in vivo with a phase contrast microscope at 40X magnification. Notes: hyalinocytes (H), semigranulocytes (SG) and granulocytes (G).

Figura 1. Hemocitos de A. *Dilocarcinus pagei* B. *Zilchiopsis collastinensis*, C. *Aegla uruguayana* y D. *Macrobrachium borellii* observados en vivo con un microscopio de contraste de fases a 40 aumentos. Notas: hialinocitos (H), semigranulocitos (SG) y granulocitos (G).

Table 2. Morphological characterization of hemocyte populations by light microscopy. Notes: Values are means ± standard deviation (SD). Hyalinocytes (H), semigranulocytes (SG) and granulocytes (G).

Tabla 2. Caracterización morfológica de las poblaciones de hemocitos mediante microscopía óptica. Notas: Los valores son medias ± desviación estándar (DE). Hialinocitos (H), semigranulocitos (SG) y granulocitos (G).

	Cell size (µm)		
	H	SG	G
Dilocarcinus pagei	11.3 ± 0.7	12.8 ± 0.2	15.2 ± 0.8
Zilchiopsis collastinensis	19.9 ± 2.2	21.3 ± 1.9	22.8 ± 3.5
Aegla uruguayana	14.4 ± 1.7	15.7 ± 2.2	20.8 ± 2.2
Macrobrachium borellii	14.2 ± 1.5	16.2 ± 0.9	20.1 ± 2.2
p-value	3.56×10^{-7}	9.95×10^{-13}	3.18×10^{-7}

the largest H size, while *D. pagei* showed the smallest. A similar pattern was observed for SG, where *Z. collastinensis* presented the largest size and *D. pagei* the smallest (Figure 2b). As for G, *A. uruguayana*, *M. borellii* and *Z. collastinensis* showed the largest sizes, with no statistical differences among them, while *D. pagei* exhibited the smallest size (Figure 2c). Total number of circulating hemocytes was approximately 4.3 ± 0.58 $x10^6$ cells/mL (*D. pagei*), 2.3 ± 0.33 $x10^6$ cells/mL (*Z. collastinensis*); 3.1 ± 0.56 $x10^6$ cells/mL (*A. uruguayana*) and 2.6 ± 0.25 $x10^6$ cells/mL (*M. borellii*) (Figure 3). Statistical analyses showed significant differences between some species ($p < 0.05$). In relation to THC, there were significant differences between *D. pagei* and *A. uruguayana* and between these and *Z. collastinensis* and *M. borellii*. However, these last two species showed no differences between them.

Flow cytometry

Based on their relative cell sizes and internal complexities (Figure 4), three cell types were identified in circulating hemocytes by flow cytometry using forward scatter (FSC) and side scatter (SSC) parameters. Hyalinocytes

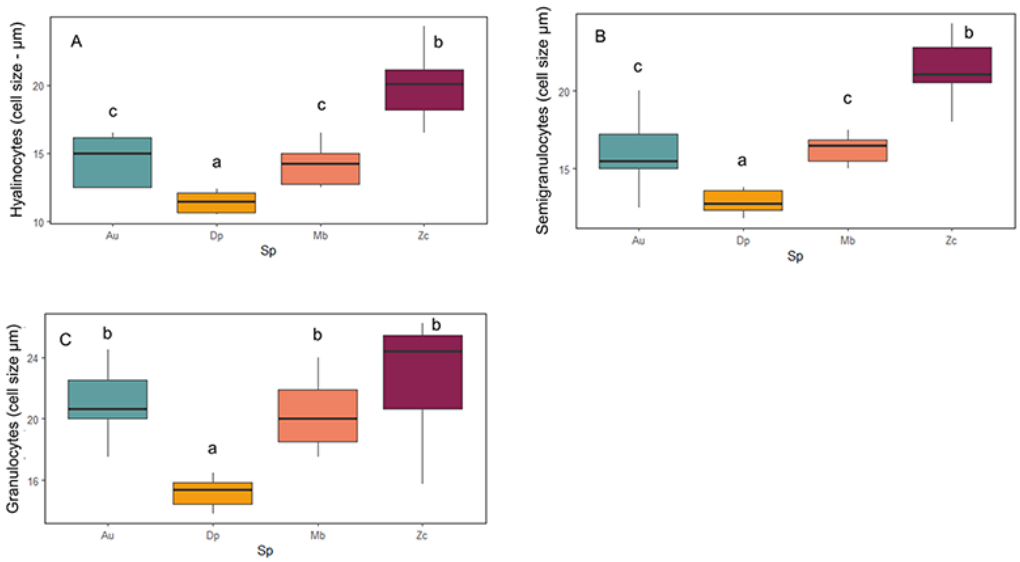

Figure 2. Comparison of the size of hemocytes in hemolymph samples from *Dilocarcinus pagei* (Dp), *Zilchiopsis collastinensis* (Zc), *Aegla uruguayana* (Au) and *Macrobrachium borellii* (Mb). The central horizontal line within the box represents the median, and the whiskers (dashed lines) extending from the box indicate the minimum and maximum values (standard deviations). Different lowercase letters indicate statistically significant differences ($p < 0.05$). Note: A. hyalinocytes, B. semigranulocytes and C. granulocytes.

Figura 2. Comparación del tamaño de los hemocitos en muestras de hemolinfa de *Dilocarcinus pagei* (Dp), *Zilchiopsis collastinensis* (Zc), *Aegla uruguayana* (Au) y *Macrobrachium borellii* (Mb). La línea horizontal central dentro del recuadro representa la mediana, y los bigotes (líneas discontinuas) que se extienden desde el recuadro indican los valores mínimo y máximo (desviaciones estándar). Letras minúsculas diferentes indican diferencias estadísticamente significativas ($p < 0.05$). Nota: A. hialinocitos, B. semigranulocitos y C. granulocitos.

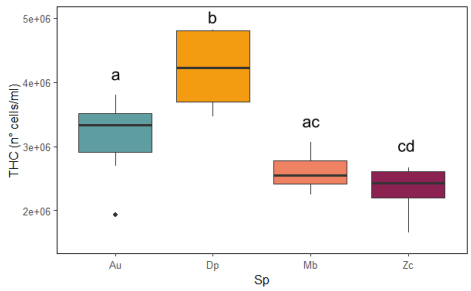

Figure 3. Total haemocyte counts (THC) of *Dilocarcinus pagei* (Dp), *Zilchiopsis collastinensis* (Zc), *Aegla uruguayana* (Au) and *Macrobrachium borellii* (Mb) are shown as mean ± SD. Different lowercase letters indicate statistically significant differences ($p < 0.05$).

Figura 3. Los recuentos totales de hemocitos (RTH) de *Dilocarcinus pagei* (Dp), *Zilchiopsis collastinensis* (Zc), *Aegla uruguayana* (Au) y *Macrobrachium borellii* (Mb) se presentan como media ± DE. Letras minúsculas diferentes indican diferencias estadísticamente significativas ($p < 0.05$).

were the most abundant cell type in circulating hemocytes of *D. pagei*, *Z. collastinensis* and *A. uruguayana* and accounted for approximately 89.87%, 84.15% and 92.33%, respectively (Figure 4a-c). Meanwhile, SG were the most abundant cell type in circulating hemocytes of *M. borellii* (68.19%) (Figure 4d). Finally, G represented the lowest percentage of circulating hemocytes in the four species (1.87% in *D. pagei*, 5.23% in *Z. collastinensis*, 0.98% in *A. uruguayana* and 2.41% in *M. borellii*). Table 3 summarizes the characteristics of the cytometric analysis (percentage of each cell type and cell density).

DISCUSSION

This study presents the first characterization of the hemocytes of four species of decapod crustaceans inhabiting streams and shallow lakes related to the Paraná River: *Dilocarcinus pagei* and *Zilchiopsis collastinensis* (family Trichodactylidae), *Aegla uruguayana* (family Aeglidae), and *Macrobrachium borellii* (family Palaemonidae). Three types of hemocytes were identified using flow cytometry and light microscopy, and they were found to be morphologically consistent with generally accepted crustacean hemocyte classifications. Flow cytometry (FC) enabled effective

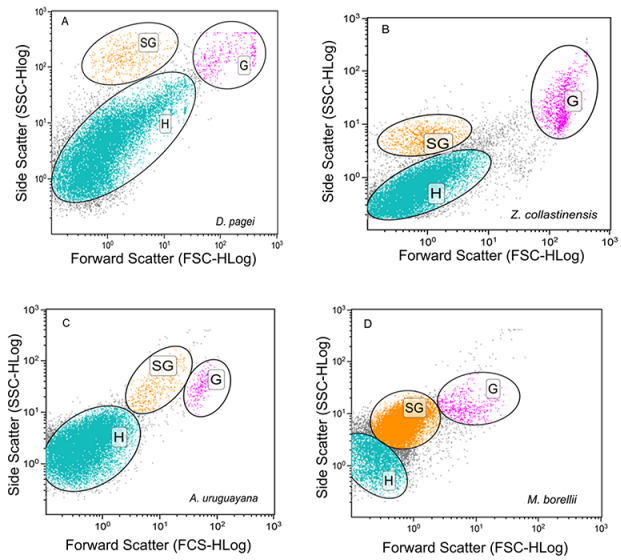

Figure 4. Scatter plot showing cell size (forward scatter, FSC) and internal complexity (side scatter, SSC) of hemocytes from A. *Dilocarcinus pagei*, B. *Zilchiopsis collastinensis*, C. *Aegla uruguayana* and D. *Macrobrachium borellii* by flow cytometry. Three categories of hemocytes can be differentiated: hyalinocytes (H), semigranulocytes (SG) and granulocytes (G).

Figura 4. Diagrama de dispersión que muestra el tamaño celular (dispersión frontal, FSC) y la complejidad interna (dispersión lateral, SSC) de los hemocitos de A. *Dilocarcinus pagei*, B. *Zilchiopsis collastinensis*, C. *Aegla uruguayana* y D. *Macrobrachium borellii* mediante citometría de flujo. Pueden diferenciarse tres categorías de hemocitos: hialinocitos (H), semigranulocitos (SG) y granulocitos (G).

Table 3. Cytometric characterization of hemocyte populations for the different studied species. Notes: hyalinocytes (H), semigranulocytes (SG) and granulocytes (G). THC: total hemocyte count.

Tabla 3. Caracterización citométrica de las poblaciones de hemocitos. Notas: hialinocitos (H), semigranulocitos (SG) y granulocitos (G). RTH: recuento total de hemocitos.

	Percentage (%)			THC
	H	SG	G	(x10^6 cells/mL)
Dilocarcinus pagei	89.87 ± 2.3	2.22 ± 1.50	1.87 ± 0.90	4.3 ± 0.58
Zilchiopsis collastinensis	84.15 ± 2.28	4.81 ± 2.22	5.23 ± 2.50	2.3 ± 0.33
Aegla uruguayana	92.33 ± 3.38	2.38 ± 1.97	0.98 ± 0.62	3.1 ± 0.56
Macrobrachium borellii	22.04 ± 5.13	68.19 ± 7.64	2.41 ± 1.17	2.6 ± 0.25
p-value				3.5 x10^{-8}

discrimination between hemocyte types by morphology and light scattering, which was confirmed with microscopy. The three cell types, differing in size and granularity, were separated using FSC (forward scatter) and SSC (side scatter) to represent cell size and granularity, respectively. While microscopy provides precise data, its time and labor demands restrict the number of cells examined. Flow cytometry, in contrast, allows for rapid analysis of larger sample sizes and minimizes subjective interpretation through automation. Our findings support previous suggestions (Sequeira *et al*., 1995; Taylor *et al*., 2014) that FC is preferable for reliable hemocyte identification and counting. Flow cytometry has been widely used to analyze various crustacean hemocytes (Cárdenas *et al*., 2000; Ding *et al*., 2012; Du *et al*., 2012; Koiwai *et al*., 2017). However, many studies rely primarily on cell size and granularity, often without incorporating microscopic verification. In the four crustacean species studied, G showed higher granularity values than SG and H, with the latter being smaller and less complex. In the two crab species (*D. pagei* and *Z. collastinensis*), cells were easily distinguishable under light microscopy based on the presence or absence of granules. By contrast, *A. uruguayana* and *M. borellii* showed higher refringence in the granules of G and SG than in crab cells. Our results align with findings by Taylor *et al*. (2014) and Li *et al*. (2018), who successfully identified three cell types—hyaline, semigranular, and granular cells—by combining flow cytometry and microscopic analysis. Taylor *et al*. (2014) observed these types based on light scattering and noted hyaline cells had smaller cell areas. Similarly, Li *et al.* (2018) identified all three cell types, though H were seldom detected with flow cytometry and appeared primarily through microscopic observation.

Mauro *et al*. (2022) found that in *Cherax quadricarinatus* and *Cherax destructor*, H were the predominant cell type, constituting 44.3% and 39% of circulating hemocytes, respectively. Our studies evidenced that *D. pagei*, *Z. collastinensis* and *A. uruguayana* showed high proportions of H, with 89.87%, 84.15 and 92.33% respectively. In rock lobster (*Jasus edwardsii*), H reached 78% of total hemocytes (Day *et al*., 2019). Some researchers, such as Zhang *et al*. (2006), suggest that H may represent an immature form of SG, citing evidence from *Fenneropenaeus chinensis* where intermediates between H and SG were observed. In contrast, others, such as Sung and Sun (2002), argue that hyaline and granular cells (SG and G) are separate, with distinct morphological and functional roles. According to Aguirre-Guzmán *et al*. (2009), in penaeid shrimps, SG constitute approximately 75% of circulating hemocytes, while G represent between 10 and 20%. In our work, *M. borellii* showed a high percentage of SG (68.19%) although the level of G was lower than that reported by these authors (2.41%). In other species of the genus *Macrobrachium*, such as *M. acanthurus* and *M. rosenbergii*, SG represented 60% and 54% and H represented 20% and 17% of circulating hemocytes (Gargioni & Barracco, 1998), which coincides with what was observed in *M. borellii*. A wide variety of hemocytes have been documented in invertebrates, with an increase in diversity and specialization based on body size and anatomical complexity (Buchmann,

2014). In crustaceans with less complex immune systems, such as certain copepods, hemocytes have more basic immune functions. By contrast, in more advanced crustaceans, such as crabs and shrimp, hemocytes display greater specialization and engage in complex immune functions, including phagocytosis and the production of antimicrobial peptides (Söderhäll & Söderhäll, 2022). Differences in hemocyte type distribution among crustacean species are significant. However, the morphological criteria for identifying crustacean hemocytes remain somewhat subjective, complicating accurate classification (Liu *et al*., 2021). Recent approaches, like RNA-based molecular analysis, have shown promise in advancing hemocyte classification, as demonstrated by a study on *Marsupenaeus japonicus*, where hemocytes were classified into six types based on transcriptional profiles (Koiwai *et al*., 2017). Total hemocyte count (THC) is a key hemolymph parameter to assess the health status of organisms (Smith *et al*., 1995; Jussila *et al*., 1997; Cheng & Chen, 2001; Mauro *et al*., 2022). The data in our study come from animals not subjected to specific stress, allowing these results to serve as a baseline for comparison with stressed individuals and to establish a welfare reference. THC values varied among species and aligned with those reported for *Jasus edwardsii* (3.1 x10^6 cells/mL) by Day *et al*. (2019) and *Carcinus maenas* (4.1 x10^6 cells/mL) by Frizzera *et al.* (2022), similar to *A. uruguayana* and *Z. collastinensis*. However, our THC values were lower than the 10.92 x10^6 cells/mL reported by Zhou *et al*. (2018). *D. pagei* had the highest THC (4.2x10^6 cells/mL), consistent with the range for *Cancer borealis* and *Cancer pagurus* (Parrinello *et al*., 2015). Crustaceans show high THC variability due to both intrinsic and extrinsic factors (Johansson *et al*., 2000). Differences in THC and hemocyte proportions among crustaceans may result from factors like seasonal changes, habitat, molting, diet, climate, and stress. In this study, even species of the same family, such as *D. pagei* and *Z. collastinensis*, showed marked differences in THC (4.2 x10^6 vs. 2.2 x10^6 cells/mL). In addition, the hemocytes of *Z. collastinensis* were also higher than those of *D. pagei*, probably reflecting adaptations to their different habitats: *D. pagei* inhabits aquatic vegetation, whereas *Z. collastinensis* occupies burrows in fine sediments. The innate immune system is crucial for invertebrates, including crustaceans (Lee & Söderhäll, 2002), with circulating hemocytes playing a key role in immune defense. Identifying and classifying hemocytes is essential for understanding specific immune responses (Ding *et al*., 2012) and serves as a physiological marker for detecting stress in these animals. Frizzera *et al*. (2022) suggested that *C. maenas* exhibited a differential hemocytic response to environmental variability, with flow cytometry revealing significant differences in hemocyte percentages between sampling sites (clean *vs*. contaminated). These variations in total hemocyte counts and percentages may be linked to environmental stress.

CONCLUSIONS

To date, there are no studies assessing hemolymphatic cell parameters related to the health status of the four native crustacean species studied. This research provides the first description of hemocyte types and total hemocyte counts based on morphological and cytometric characteristics. In all four species, three cell types were identified: hyalinocytes were predominant in *D. pagei*, *Z. collastinensis* and *A. uruguayana*, while semigranulocytes were more abundant in *M. borellii*. Granulocytes were present in lower proportion in all species. In addition, *Z. collastinensis* and *M. borellii* showed the lowest number of cells per ml of hemolymph, while *D. pagei* and *A. uruguayana* showed higher counts. Our results highlight the usefulness of flow cytometry to differentiate and quantify hemocytes in these species, positioning it as a valuable tool to assess physiological status. Future studies could employ these cellular parameters as early indicators of environmental stress, and comparison with data from contaminated sites could identify reliable biomarkers of physiological damage.

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