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Article

Ecological and reproductive biology of two sympatric species of *Hyalella* (Crustacea, Amphipoda, Hyalellidae) from southern Brazil

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ABSTRACT. In this work, the population dynamics and reproductive aspects of two sympatric *Hyalella* species in a river spring were analyzed in the northwestern state of Rio Grande do Sul, Brazil. The samplings were performed by one person, for ten minutes and using a hand net for one-year period (March 2018 to April 2019) and transported to the laboratory. All individuals sampled were sexed, measured, and separated by cephalothorax length (CC) size classes. *Hyalella gauchensis* Streck & Castiglioni, 2017 was 22.7 times more frequent and showed significantly greater mean cephalothorax when compared to *Hyalella longipropodus* Limberger, Graichen & Castiglioni, 2021. Males had a larger body size (CC) than females, although they occur in smaller numbers in both species. The total frequency distribution in CC size classes was bimodal in males and juveniles, and polimodal in females of *H. gauchensis.* In *H. longipropodus* the distribution as bimodal in males, females and juveniles. Both *H. gauchensis* and *H. longipropodus* had their breeding season and recruitment during the colder seasons of the year (fall and winter, respectively). However, there was a seasonal temporal separation of the intensity peaks between the two species, which could indicate different strategies and/or evolutionary adaptations for their coexistence.

KEYWORDS. Freshwater Amphipoda, population dynamics.

RESUMO. Ecologia e biologia reprodutiva de duas espécies simpátricas de *Hyalella* (Crustacea, Amphipoda, Hyalellidae) do sul do Brasil. Neste trabalho foram analisadas a biologia populacional e aspectos reprodutivos de duas espécies simpátricas de *Hyalella* em uma nascente encontrada na região noroeste do estado do Rio Grande do Sul, Brasil. As amostragens foram realizadas por uma pessoa durante dez minutos com puçá no período de um ano (Março/2018 a Abril/2019) e transportadas ao laboratório. Todos os indivíduos amostrados foram sexados, mensurados e separados por classes de tamanho de comprimento do cefalotórax (CC). *Hyalella gauchensis* Streck & Castiglioni, 2017 foi 22,7 vezes mais frequente e apresentou comprimento do cefalotórax médio significativamente superior a *Hyalella longipropodus* Limberger, Graichen & Castiglioni, 2021. Machos apresentaram maior tamanho corpóreo (CC) que as fêmeas, embora ocorram em menor número em ambas as espécies. A distribuição de frequência total em classes de tamanho de CC foi bimodal em machos e juvenis e polimodal em fêmeas de *H. gauchensis.* Em *H. longipropodus* a distribuição foi bimodal em machos, fêmeas e juvenis. Tanto *H. gauchensis* quanto *H. longipropodus* tiveram seu pico reprodutivo e de recrutamento durante as estações mais frias do ano (outono e inverno, respectivamente), entretanto, houve separação temporal sazonal dos picos de intensidade entre as duas espécies, o que poderia indicar diferentes estratégias e/ou adaptações evolutivas para a coexistência das mesmas.

PALAVRAS-CHAVE. Amphipoda dulcícola, dinâmica populacional.

The benthic fauna of freshwater ecosystems includes important members in the form of crustacean species belonging to the genus *Hyalella* Smith, 1874. These species are known to facilitate the flow of energy within the aquatic environment, thereby playing a significant role in the trophic chain (Wen, 1992; Väinölä *et al*., 2008). Amphipod species generally exhibit a limited geographic distribution, likely due to the absence of a dispersed life stage (Barnard & Karaman, 1983). The phenomenon of endemism is particularly pronouncing among freshwater-dwelling species, especially those that inhabit lakes, ponds, and underground environment (Väinölä *et al*., 2008). This is observed in case of amphipod belonging to the genus *Hyalella*.

Hyalella species are geographically restricted to the Nearctic and Neotropical biogeographical regions, being commonly found in North America, also occurring in Central and South America (González & Watling, 2002; González *et al.*, 2006). They are typically associated with macrophytes and can be found swimming in the water column or burrowing in the sediment of permanent reservoirs, springs, lakes, and streams (Grosso & Peralta, 1999; Bueno *et al*., 2014; Streck *et al*., 2017). Some species, such as *H. georginae* Streck & Castiglioni, 2017, *H. palmeirensis* Streck-Marx & Castiglioni, 2020, and *H. longipropodus* Limberger, Graichen & Castiglioni, 2021, are limited to a single pond or stream (Streck *et al*., 2017; Streck-Marx & Castiglioni, 2020; Limberger *et al*., 2021). Furthermore, some *Hyalella* species are sympatric, such as *H. pleoacuta* González, Bond-Buckup & Araujo, 2006 and *H. castroi* González, Bond-Buckup & Araujo, 2006 (Castiglioni & Bond-Buckup, 2007, 2008a), *H. virgineae* Penoni & Bueno, 2021 and *H. bal*a Lares, Penoni & Bueno, 2021 (Penoni *et al*., 2021), as well as *H. longipropodus* and *H. gauchensis* Streck & Castiglioni, 2017 (Limberger *et al*., 2021).

Currently, there are approximately 98 species of *Hyalella* described for the Americas, of which 39 occur in Brazil (Bueno *et al*., 2019; Peralta & Isa Miranda, 2019; Rogers *et al*., 2020; Streck-Marx & Castiglioni, 2020; Reis *et al*., 2020; Talhaferro *et al*., 2021; Penoni *et al*., 2021; Jaume *et al*., 2021; Rangel *et al*., 2022; Limberger *et al*., 2022; Isa-Miranda & Peralta, 2022; Marrón-Becerra & Hermoso-Salazar, 2022). Studies on the population biology and reproductive strategies of *Hyalella* in Brazil have been conducted with *H. bonariensis* Bond-Buckup, Santos & Araujo, 2006 (Castiglioni *et al*., 2016, 2018), *H. georginae* and *H. gauchensis* (Ozga & Castiglioni, 2017; Ozga *et al*., 2018), and *H. palmeirensis* Streck-Marx & Castiglioni, 2020 (Castiglioni *et al*., 2020). Reproductive aspects have also been analyzed in populations of *H. carstica* Bastos-Pereira & Bueno, 2012 (Torres *et al*., 2015) and *H. longistila* Faxon, 1876 (Bastos-Pereira & Bueno, 2016). Recently, Zepon *et al.* (2021) investigated the distribution and new information on the natural history and habitat of the troglobitic amphipod *H. veredae* Cardoso & Bueno, 2014.

However, studies on the ecological interactions among sympatric species of the genus *Hyalella* are limited to research on population and reproductive biology of *H. pleoacuta* and *H. castroi* in the state of Rio Grande do Sul (Castiglioni & Bond-Buckup, 2007; Castiglioni *et al*., 2007; Castiglioni & Bond-Buckup, 2008a, b; Castiglioni & Bond-Buckup, 2009). According to Castiglioni & Bond-Buckup (2008a), it is suggested that the co-occurrence of *Hyalella* species is possible due to subtle differences in traits of their life history, especially body size and reproductive strategies. Thus, understanding the biological cycle and, in particular, assessing the population and reproductive dynamics can help in interpreting the conservation status of populations and assist in developing policies for the preservation of freshwater ecosystems. Furthermore, Penoni *et al.* (2021) present some data on the population biology and reproduction of two sympatric species, *H. virgineae* and *H. bala* from Parque Estadual da Serra do Mar, in the state of São Paulo.

In this sense, the purpose of this study is to characterize and compare the population and reproductive biology of two species of the genus *Hyalella* (*H. gauchensis* and *H. longipropodus*), which coexist in a spring located in the northwestern region of the state of Rio Grande do Sul, in southern Brazil.

MATERIALS AND METHODS

The fieldwork was developed in a small spring (27°51'38"S – 53°15'11"W) of the watershed of the Rio da Várzea (Uruguay Basin), on private property in the region of Capão Alto, District of Santa Rosa, municipality of Palmeira das Missões, northwest region of state of Rio Grande do Sul, Brazil, with an altitude of 526 m above sea level (Fig. 1). The spring had no riparian forest, but there were macrophytes of the genus *Polygonum* Linnaeus, 1753 and *Lemna* Linnaeus, 1753 and deep about 30 cm to 50 cm. The associated fauna found with *Hyalella* species was basically composed of macroinvertebrates, such as dragonflies and mayflies larvaes, trichopterans, aquatic beetles and leeches. Furthermore, at certain times of the year tadpoles, small lambari fish and some birds were observed on the banks of the spring. It should be noted that the source is located at about 200 m from the residence of the rural property and the water is collected to be used for human consumption. It should be noted that on the rural property there is no soybean plantation area, but dairy cattle farming. However, in the surroundings there are several properties that cultivate especially soybeans, wheat and oats. The climate is subtropical, with hot summers, rainfall distributed throughout the year and mean temperature of 22º C in the hottest period (Moreno, 1961). This region has a large part of its territory occupied by soybean and wheat crops with alternate planting according to the respective harvest and intercrop seasons (IBGE, 2019).

The *Hyalella* samples were collected monthly for one year (from April 2018 to March 2019) from various microhabitats, including macrophytes and sediment. Sampling was performed randomly by a single person using a hand net with a mesh size of 250 µm for 10 minutes. The samples were stored in plastic bags and placed in a thermal box with ice for proper transportation to the laboratory. Amphipods were visually inspected in the field to identify ovigerous females and pairs in pre-copulatory behavior. These individuals were individualized and preserved in microtubes with 70% ethanol. The sampling of *Hyalella* specimens was accompanied by the measurement of environmental variables such as air and water temperature, and pH. These measurements were taken monthly using a digital thermometer (Incoterm) and a pH meter (Hanna), respectively. Environmental variables measured monthly at the study site are showed in Tab. I.

In the laboratory, the specimens were identified using a stereomicroscope (OLYMPUS, model SZ2-LGB), based on characteristics such as the shape, number, and arrangement of the setae of buccal appendages, antennae,

Fig. 1. Spring of *Hyalella gauchensis* Streck & Castiglioni, 2017 and *H. longipropodus* Limberger, Graichen & Castiglioni, 2021 were sampled in sympatry in Capão Alto, District of Santa Rosa, municipality of Palmeira das Missões, northwest region of state of Rio Grande do Sul, southern Brazil. This spring is the type locality of *H. longipropodus*.

gnathopods, uropods, and telson, following the criteria established by Streck *et al.* (2017) and Limberger *et al.* (2021). Subsequently, individuals of *H. gauchensis* and *H. longipropodus* were separated into four distinct groups: males (individuals with evident development of gnathopod 2), females (individuals with oostegites and a small gnathopod 2), ovigerous females (females carrying eggs or juveniles inside the marsupium), and juveniles (individuals without secondary sexual characteristics such as the development of gnathopod 2 or oostegites presence) (Castiglioni & Bond-Buckup, 2007). All specimens were then measured using an OLYMPUS microscope (model CZ2-LGB) with a micrometer eyepiece to determine the cephalothorax length (CL) in mm (Borowsky, 1991; Castiglioni & Bond-Buckup, 2008a), which was taken from the anterior margin of the rostrum to the posterior margin of the cephalothorax. The CL was used as a proxy for animal size, as it has been shown to have a positive correlation with the total length of *H. azteca* (Saussure, 1858) (Edwards & Coweel, 1992; Pickard & Benke, 1996). After analysis, the specimens were deposited in the LTA scientific collection (*H. gauchensis* - LTA300 to LTA311; *H. longipropodus* – LTA312 to LTA323).

The study analyzed various population and reproductive parameters of *H. gauchensis* and *H. longipropodus*, including abundance, body size of males and females, sexual maturity (defined as the size of the smallest ovigerous female and the smallest male and female in pre-copulatory behavior), size distribution, sex ratio, reproductive period (defined as the frequency of ovigerous females and pairs), and recruitment (juveniles frequency). These parameters were analyzed seasonally, with seasons defined as autumn (April to June),

Tab. I. Abiotic data (pH, water temperature and air temperature) by sampling months of *Hyalella gauchensis* Streck & Castiglioni, 2017 and *H. longipropodus* Limberger, Graichen & Castiglioni, 2021 in the northwest region of state of Rio Grande do Sul, Southern Brazil.

Month	pH	Water temperature (º C)	Air temperature (º C)
Apr/2018	6.68	21.7	22.1
May/2018	6.55	18.0	21.2
Jun/2018	6.61	13.0	9.00
Jul/2018	6.59	14.5	13.9
Aug/2018	6.62	18.4	20.9
Sep/2018	6.50	16.3	18.1
Oct/2018	6.65	18.9	20.5
Nov/2018	6.52	18.4	21.6
Dec/2018	6.63	21.4	25.9
Jan/2019	6.80	20.9	23.5
Feb/2019	6.99	23.1	24.8
Mar/2019	6.42	22.0	26.6

winter (July to September), spring (October to December), and summer (January to March).

The abundance of each species of *Hyalella* was estimated monthly for each age group. Additionally, the proportion of *H. gauchensis* and *H. longipropodus* was calculated by month and a goodness-of-fit test (χ^2) was used to evaluated the proportion of species (α= 0.05) (Zar, 2010).

The body size of the amphipods was assessed by determining the minimum, maximum, and mean (± standard deviation) cephalothorax length (CL) of males, females, ovigerous females, and juveniles of both *Hyalella* species. The mean size (CL) of amphipods was compared between sex and species using a *t*-test (α=0.95) (Zar, 2010).

The sexual maturity of both *Hyalella* species was assessed using two methods: (a) analyzing the cephalothorax length of the smallest males and females found in pre-copulatory behavior, as described by Borowsky (1991), Castiglioni & Bond-Buckup (2008a), and Ozga & Castiglioni (2017); and (b) determining the size of the smallest ovigerous female in the population, following the methods described by Castiglioni & Bond-Buckup (2008a) and Ozga & Castiglioni (2017).

The frequency distribution by size class was analyzed for each group (juveniles, males and females) in both *Hyalella* species, both for the total population and seasonally. This analysis is important to monitor temporal changes in the age frequency distribution of populations and to observe the seasonality of processes such as reproduction and recruitment. The population was grouped into 0.05 mm size classes (20 cephalothorax length size classes) after measurement, and the number of size classes was determined by one-quarter of the standard deviation of the cephalothorax length of the amphipods sampled throughout the study (Markus, 1971). The normality of frequency distributions was analyzed by the Shapiro-Wilk test with a significance level of α=0.05 (Zar, 2010).

The total sex ratio, as well as monthly, seasonal, and size-specific ratios, were estimated by dividing the total number of males by the total number of females (males: females). To determine if the sex ratio followed a 1:1 ratio, we performed a goodness-of-fit test (χ2) with a significance level of 5% (Zar, 2010).

The reproductive period of each species was determined monthly and seasonally based on the frequency of ovigerous females in relation to that of adult females. Additionally, we analyzed the reproductive period by examining the monthly and seasonal frequency of pairs in precopulatory behavior. We compared the proportion of ovigerous females and pairs in precopulatory behavior between months and seasons using the multinomial proportions test (MANAP; α = 0.05) (Curi & Moraes, 1981).

To analyze recruitment patterns of juveniles into the population for each *Hyalella* species, we compared the proportion of juveniles between months and seasons using the multinomial proportion test (MANAP; α = 0.05) (Curi & Moraes, 1981).

RESULTS

Population structure. The total population of *H. gauchensis* sampled during the study period accounted for 19,998 individuals, whereas for *H. longipropodus* the number of individuals collected was 880. The monthly numbers of males, females, ovigerous females and juveniles of each species are detailed in Tables II and III. In general, *H. gauchensis* showed greater abundance in all sampled

Tab. II. Number of juveniles, males, females and ovigerous females of *Hyalella gauchensis* Streck & Castiglioni, 2017 sampled monthly, sex ratio by month (males: females) and results of the goodness-of-fit test (χ^2), in the northwest region of state of Rio Grande do Sul, Southern Brazil.

Month	Juveniles	Males	Females	Ovigerous females	Total	M/F	χ2
Apr/2018	349	119	198	40	706	0.50	39.67*
May/2018	869	128	135	24	1,156	0.81	3.35
Jun/2018	624	113	244	41	1,022	0.40	74.33*
Jul/2018	1,805	375	855	140	3,175	0.38	280.58*
Aug/2018	2,231	256	775	103	3,365	0.29	341.17*
Sep/2018	1,431	224	473	140	2,268	0.37	180.79*
Oct/2018	2,975	306	725	130	4,136	0.36	259.60*
Nov/2018	479	86	221	75	861	0.29	115.45*
Dec/2018	810	145	356	33	1,344	0.37	111.49*
Jan/2019	271	154	185	39	649	0.69	12.96*
Feb/2019	353	138	204	7	702	0.65	15.27*
Mar/2019	265	156	142	51	614	0.81	3.92*
Total	12,462	2,200	4,513	823	19,998	0.41	1305.00*

Note: the "*" indicates a significant difference in the proportion between males and females (p <0.05); M= males; F= females.

months, being 22.7 times more abundant than *H. longipropodus* (χ^2= 17497,43; p<0.05) (Fig. 2).

The total frequency in cephalothorax length size classes had non-normal distribution for both *Hyalella* species (*H. gauchensis*: males - W=0.96 and females W=0.97; *H. longipropodus*: males W=0.95 and females W=0.95; p<0.05). Furthermore, the total frequency distribution was bimodal for males and juveniles, while in females it was polymodal in *H. gauchensis* (Fig. 3A). In *H. longipropodus* the distribution was bimodal for males, females and juveniles (Fig. 3B).

The size-frequency distribution analyzed seasonally showed bimodality for juveniles of *H. gauchensis*, whereas

Tab. III. Number of juveniles, males, females and ovigerous females of *Hyalella longipropodus* Limberger, Graichen & Castiglioni, 2021 sampled monthly, sex ratio by month (males: females) and results of the goodness-of-fit test (χ^2), in the northwest region of state of Rio Grande do Sul, Southern Brazil.

Months	Juveniles	Males	Females	Ovigerous females	Total	M/F	χ2
Apr/2018	7	42	60	7	116	0.63	5.73*
May/2018	10	13	13	3	39	0.93	0.04
Jun/2018	85	43	55	16	199	0.61	6.88*
Jul/2018	48	38	39	11	136	0.76	1.64
Aug/2018	29	10	11	5	55	0.63	1.38
Sep/2018	26	13	19	6	64	0.68	1.13
Oct/2018	0	10	9	4	23	0.77	0.39
Nov/2018	30	20	26	3	79	0.57	4.09*
Dec/2018	12	11	13	4	40	0.58	2.13
Jan/2019	0	6	11	0	17	0.55	1.47
Feb/2019	2	9	7	0	18	1.29	0.25
Mar/2019	28	39	19	8	94	1.44	2.18
Total	277	254	282	67	880	0.73	14.97*

Note: the "*" indicates a significant difference in the proportion between males and females (p <0.05); M= males; F= females.

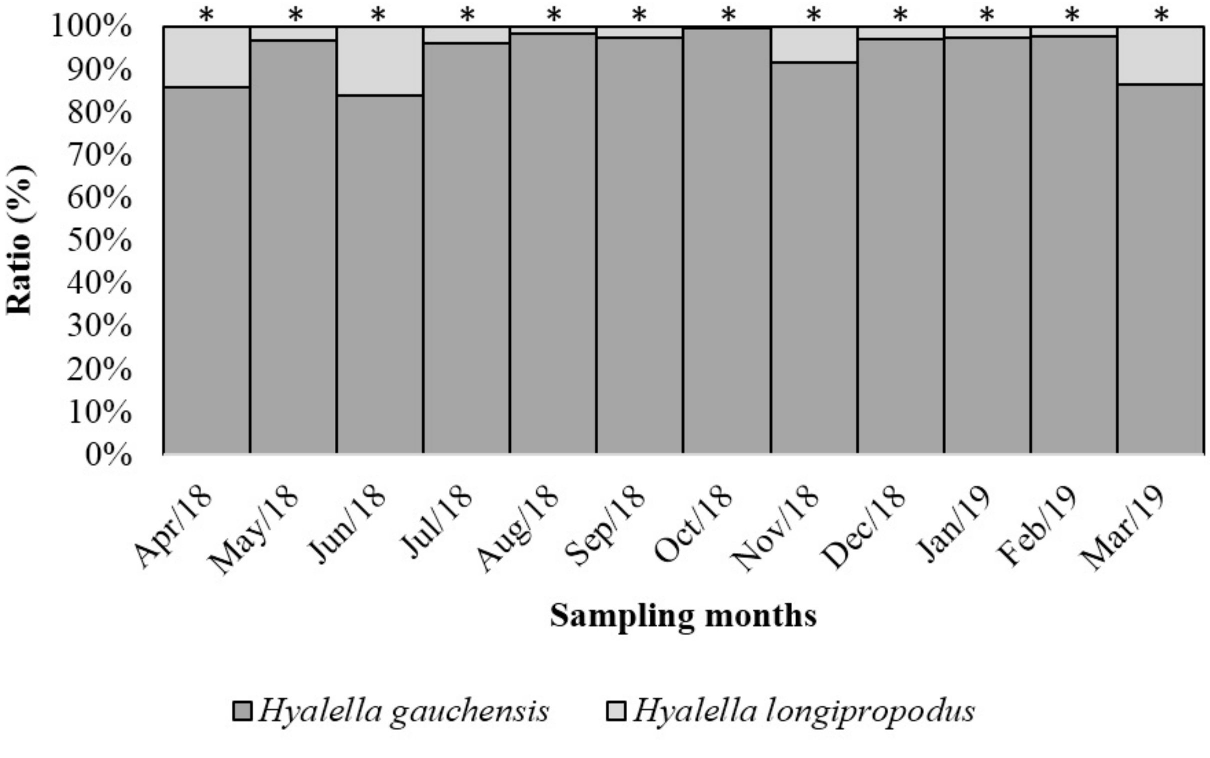

Fig. 2. Proportion of *Hyalella gauchensis* Streck & Castiglioni, 2017 and *H. longipropodus* Limberger, Graichen & Castiglioni, 2021 sampled throughout one year in the northwest region of state of Rio Grande do Sul, Southern Brazil. The "*" indicates significant differences in the proportion between the two species of *Hyalella* (p<0.05).

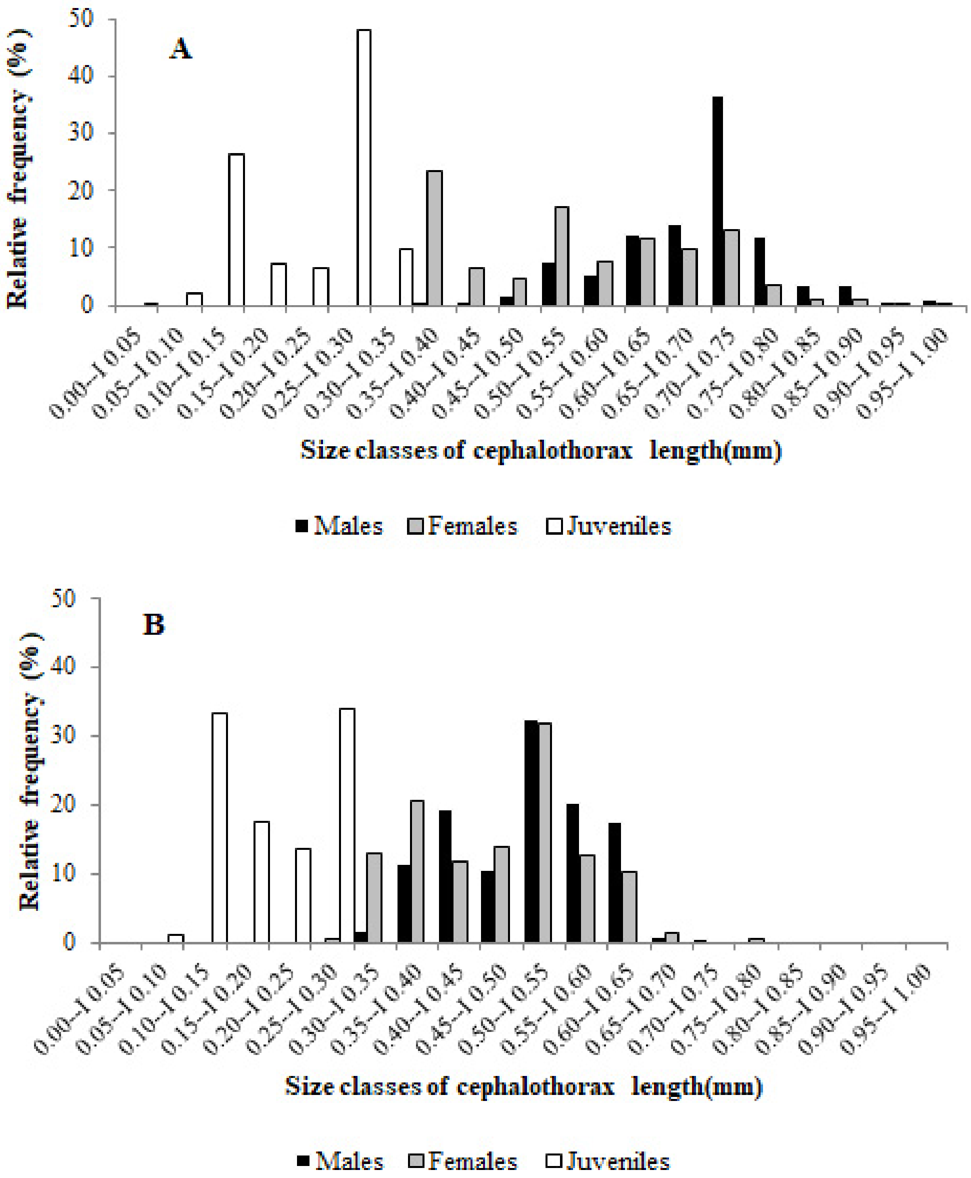

Fig. 3. Total relative frequency distribution (%) in size classes of cephalothorax length (CL) of juveniles, males and females of *Hyalella gauchensis* Streck & Castiglioni, 2017 (A) and *H. longipropodus* Limberger, Graichen & Castiglioni, 2021 (B) in the northwest region of state of Rio Grande do Sul, Southern Brazil.

for males the distribution was bimodal only during fall and unimodal the rest of the year. For females, the distribution was polymodal in the four seasons (Fig. 4). On the other hand, the size frequency distribution of *H. longipropodus* showed greater variation throughout the seasons with unimodality in winter and spring, and bimodality in summer and fall for juveniles. Males showed unimodal distribution only in fall and bimodal for the other seasons throughout the year. Females presented unimodal distribution in spring and summer and bimodal distribution for the other seasons (Fig. 5).

Body size and sexual maturity. The minimum, maximum and mean (± standard deviation) cephalothorax length of males, females, ovigerous females and juveniles of *H. gauchensis* and *H. longipropodus* are shown in Tab. IV. Males were significantly greater than females in both species (*H. gauchensis* – *t*= 44.38; *H. longipropodus* – *t*= 4.83; p<0.05; Tab. IV). Ovigerous females were significantly larger than non-ovigerous females in *H. gauchensis* (*t*= -28.73) and *H. longipropodus* (*t*= -28.73) (p<0.05; Tab. IV). Males, females, ovigerous females and juveniles of the *H. gauchensis* had mean cephalothorax length greater than *H. longipropodus* (males: *t*= 87.54, females: *t*= 87.16; ovigerous females: *t*= 10.56; juveniles: *t*= 7.89; p < 0.05; Tab. IV).

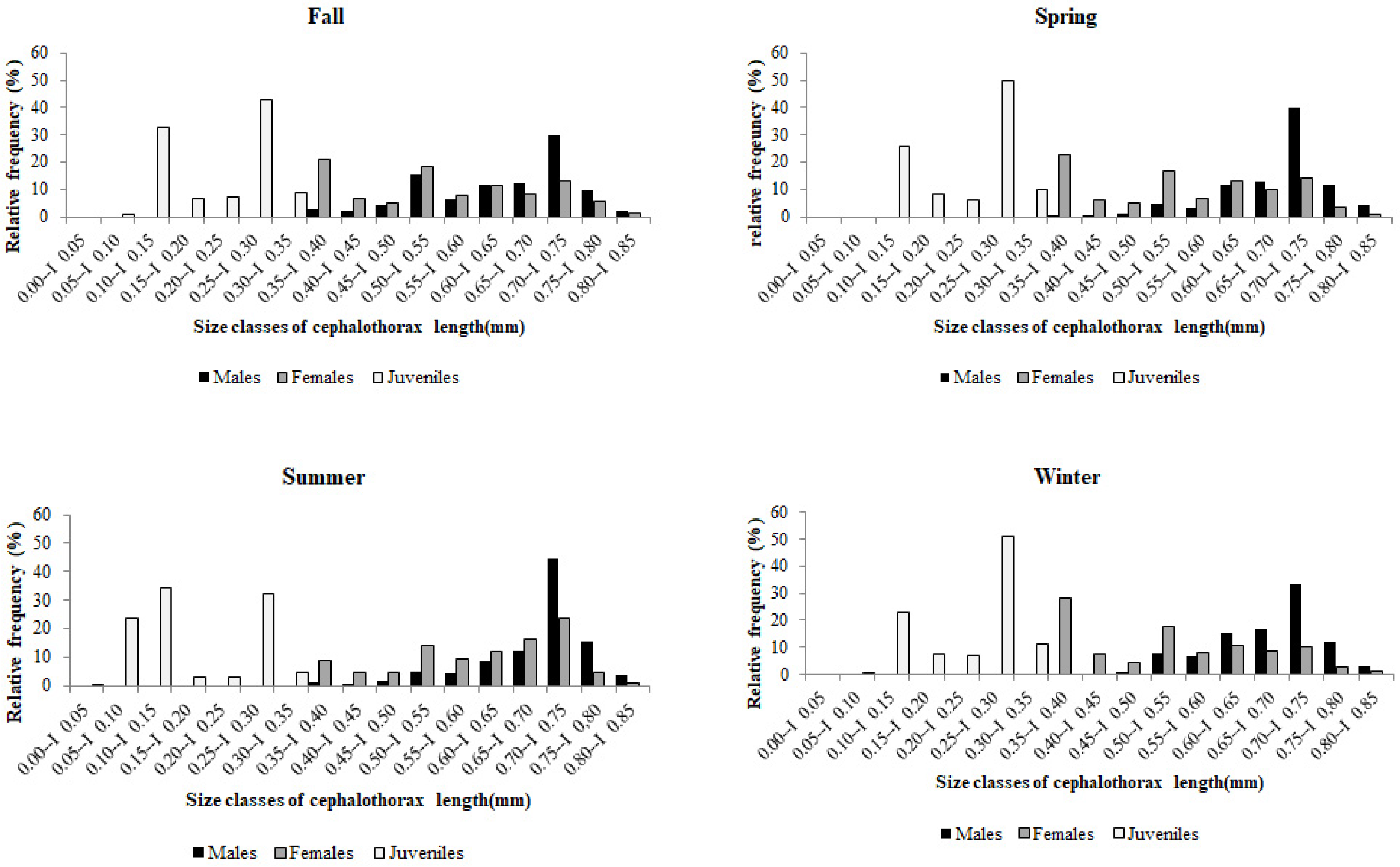

Fig. 4. Seasonal relative frequency distribution (%) in size classes of cephalothorax length (mm) of juveniles, males and females of *Hyalella gauchensis* Streck & Castiglioni, 2017 in the northwest region of the state of Rio Grande do Sul, Southern Brazil.

Tab. IV. Minimum, maximum and mean (± standard deviation) of males, females, ovigerous females and juveniles of *Hyalella gauchensis* Streck & Castiglioni, 2017 and *H. longipropodus* Limberger, Graichen & Castiglioni, 2021 in the northwest region of state of Rio Grande do Sul, Southern Brazil.

	Min-Max	Mean ± sd
Hyalella gauchensis		
Males	0.35-0.99	0.69 ± 0.11
Females	0.35-0.99	0.54 ± 0.14
Ovigerous females	0.40-0.99	0.66 ± 0.08
Juveniles	0.02-0.32	0.21 ± 0.06
Hyalella longipropodus		
Males	0.30-0.72	0.49 ± 0.08
Females	0.25-0.67	0.45 ± 0.08
Ovigerous females	0.30-0.79	0.57 ± 0.07
Juveniles	0.07-0.27	0.19 ± 0.06

Note: SE= standard error; min= minimum; max= maximum.

The size at onset of sexual maturity of *H. gauchensis* estimated based on the CL of the smallest male and female found in precopulatory behaviour was 0.47 mm for males and 0.35 mm for females; and for *H. longipropodus* was 0.42 mm for males and 0.30 mm for females. Besides, the size of the smallest ovigerous females found in the population was also used as an estimate of sexual maturity, hence, *H. gauchensis* and *H. longipropodus* are able to reproduce at CL of 0.40 mm and 0.30 mm, respectively.

Sex ratio. The total sex ratio of populations for both species favored females (*H. gauchensis* - 0.41 male: 1 female - $\chi^2=1305.00$ - $p<0.05$; *H. longipropodus* - 0.73 male: 1 female, $\chi^2=14.97$ - $p<00.5$). In *H. gauchensis* females was significantly more frequent than males along the year, except in May ($p>0.05$) (Tab. II). In *H. longipropodus*, significant differences in sex ratio were observed only in April, June and November, when females were more frequent than males ($p<0.05$) (Tab. III).

Regarding the seasonal sex ratio in *H. gauchensis*, it was observed that females were more frequent significantly than males in all seasons (fall $\chi^2=99.50$; winter $\chi^2=795.22$; spring $\chi^2=484.36$; summer $\chi^2=30.11$; $p<0.05$) (Fig. 6A). In *H. longipropodus* the sex ratio favored females in all seasons (fall $\chi^2=11.66$; winter $\chi^2=3.950$; spring $\chi^2=6.26$; $p<0.05$), except in summer when males were more abundant ($\chi^2=0.82$) ($p<0.05$) (Fig. 6B).

For the sex ratio analysis by size classes of CL in *H. gauchensis*, females predominated in intermediate classes and males predominated in larger size classes ($p<0.05$; Fig. 7A). In *H. longipropodus*, the sex ratio by size classes showed a greater variation with females being significantly more frequent in the smaller classes ($p<0.05$) (Fig. 7B). Although males were more frequent in greater size classes, the sex ratio did not differ significantly from 1:1 ($p>0.05$) (Fig. 7B).

Breeding period. Males and females in pre-copulatory behaviour were sampled all along the year, showing some frequency fluctuations in some months in *H. gauchensis* (Fig. 8A). However, *H. longipropodus* showed greater variations at the frequencies of pairs in pre-copulatory behaviour, and in some months they were not registered (Fig. 8A). Regarding seasonal analysis, males and females in precopulatory behaviour occurred in all seasons for both species (Fig. 8B). *Hyalella gauchensis* showed higher frequency of pairs in winter, with significant differences among the seasons ($p<0.05$), except between spring and summer ($p>0.05$) (Fig. 8B). In *H. longipropodus* a higher intensity of pairs was observed in fall, with significant differences between fall and winter and fall and summer ($p<0.05$) (Fig. 8B).

Ovigerous females were sampled throughout the year and showed fluctuations of their relative frequencies (Fig. 9A). Both species showed two-frequency peaks. *Hyalella gauchensis* showed greater intensity in winter, followed by spring, with significant differences for among the seasons ($p<0.05$), except fall and summer ($p>0.05$) (Fig. 9B). On the other hand, *H. longipropodus* showed greater reproductive intensity in fall, followed by winter, with significant differences between fall and spring, fall and summer, and winter and summer ($p<0.05$) (Fig. 9B).

Recruitment. Recruitment was continuous throughout the year for the species studied (Tabs II, III), as indicate by the presence of juveniles during all seasons. The highest relative frequency of juveniles of *H. gauchensis* was in winter, followed by spring, with significant differences for all seasons ($p<0.05$; Fig. 10). However, juveniles of *H. longipropodus* predominated in fall and winter (since non-significant differences were found between both frequencies), and showed lower frequencies during spring and summer ($p>0.05$; Fig. 10).

DISCUSSION

Although *H. gauchensis* and *H. longipropodus* were present throughout the year, their abundances differed greatly, with *H. gauchensis* being more abundant. The sympatric amphipod species are often found with spatial separation in microhabitats, as observed by Castiglioni & Bond-Buckup (2007) for *H. pleoacuta* and *H. castroi* and by Dick & Platvoet (1996) for *Gammarus pulex* Linnaeus, 1758 and *G. tigrinus* Sexton, 1939. We were unable to observe any such differentiation of small habitats between *H. gauchensis* and *H. longipropodus* in this study. Castiglioni & Bond-Buckup (2007) suggested that spatial separation in sympatric *Hyalella* species may indicate differences in physiological preferences or serve as a strategy to prevent competitive exclusion and facilitate species coexistence. However, *H. gauchensis* and *H. longipropodus* share many aspects of their ecological niche, especially space and food.

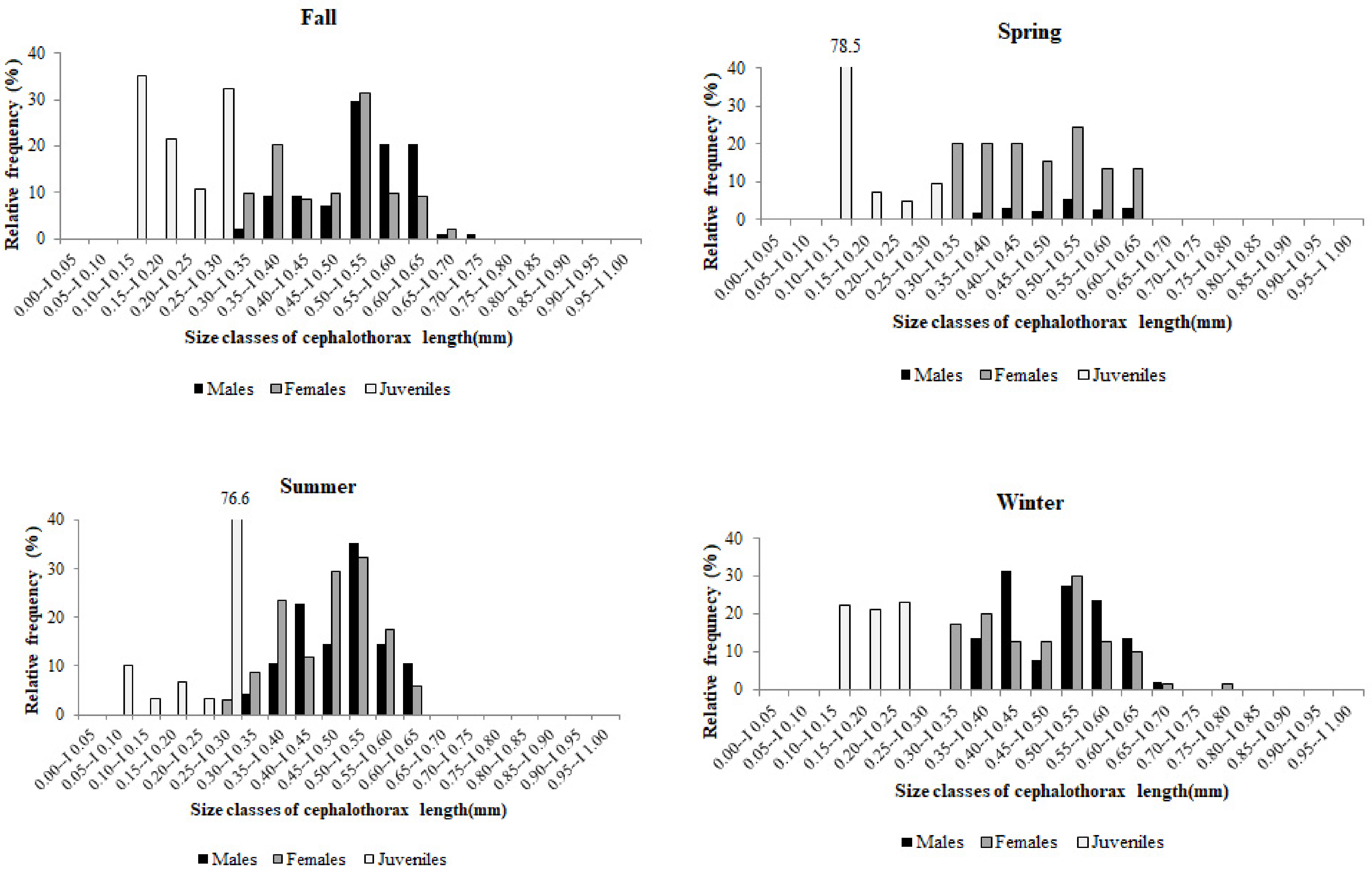

Fig. 5. Seasonal relative frequency distribution (%) in size classes of cephalothorax length (mm) of juveniles, males and females of *Hyalella longipropodus* Limberger, Graichen & Castiglioni, 2021 in the northwest region of the state of Rio Grande do Sul, Southern Brazil.

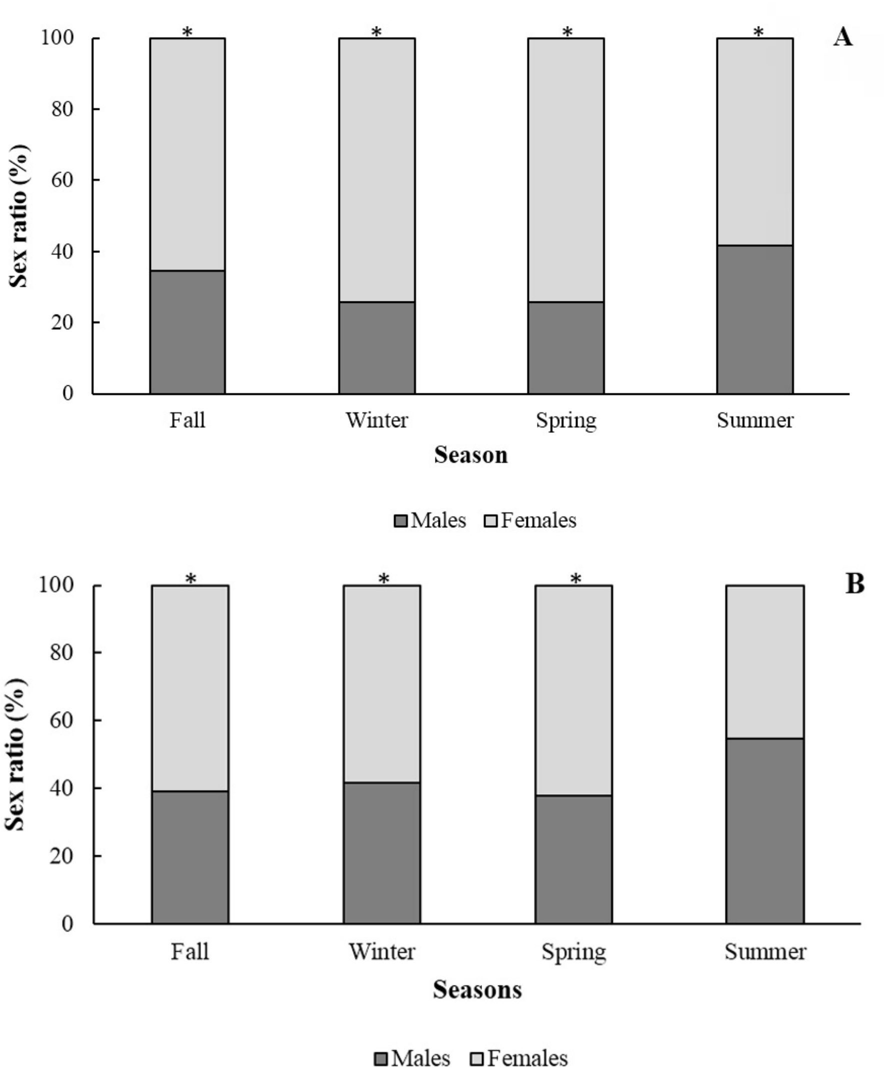

Fig. 6. Seasonal sexual proportion of *Hyalella gauchensis* Streck & Castiglioni, 2017 (A) and *H. longipropodus* Limberger, Graichen & Castiglioni, 2021 (B) in the northwest region of state of Rio Grande do Sul, Southern Brazil. "*" indicates significant differences in the proportion 1 male: 1 female (p<0.05).

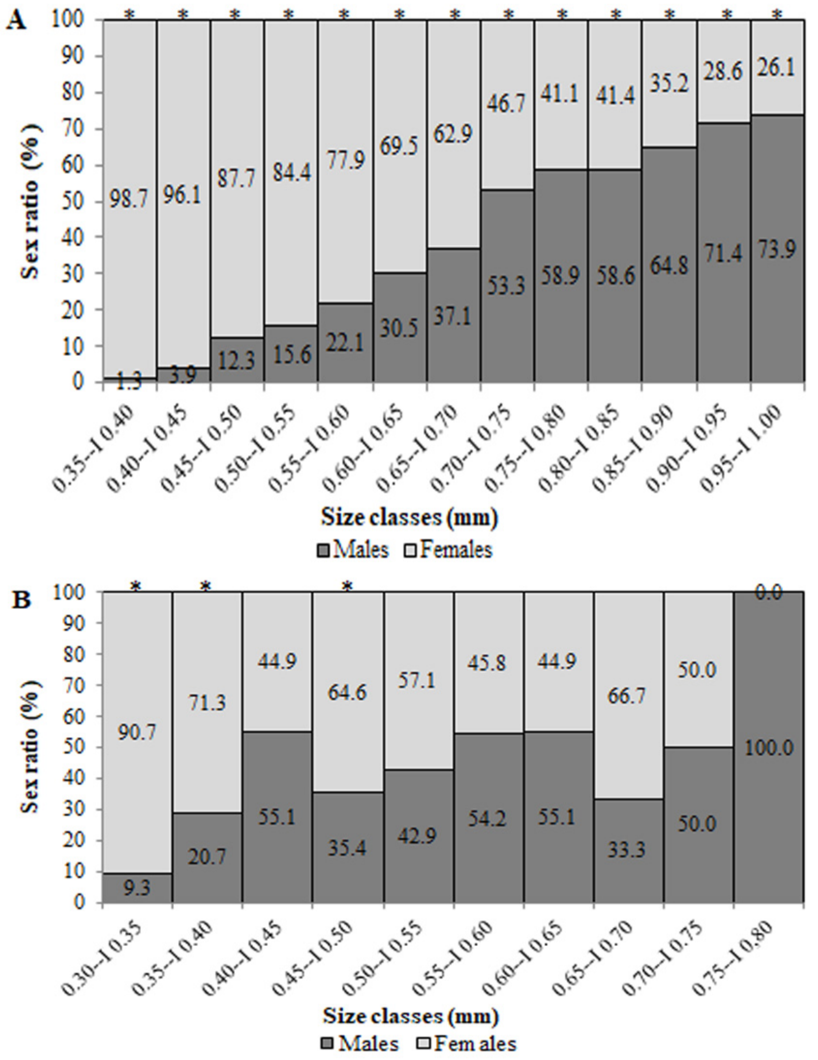

Fig. 7. Sex ratio by size classes of cephalothorax length (CL) of *Hyalella gauchensis* Streck & Castiglioni, 2017 (A) and *H. longipropodus* Limberger, Graichen & Castiglioni, 2021 (B) in northwest region of state of Rio Grande do Sul, Southern Brazil. "*" indicates significant differences in the sex ratio (p<0.05).

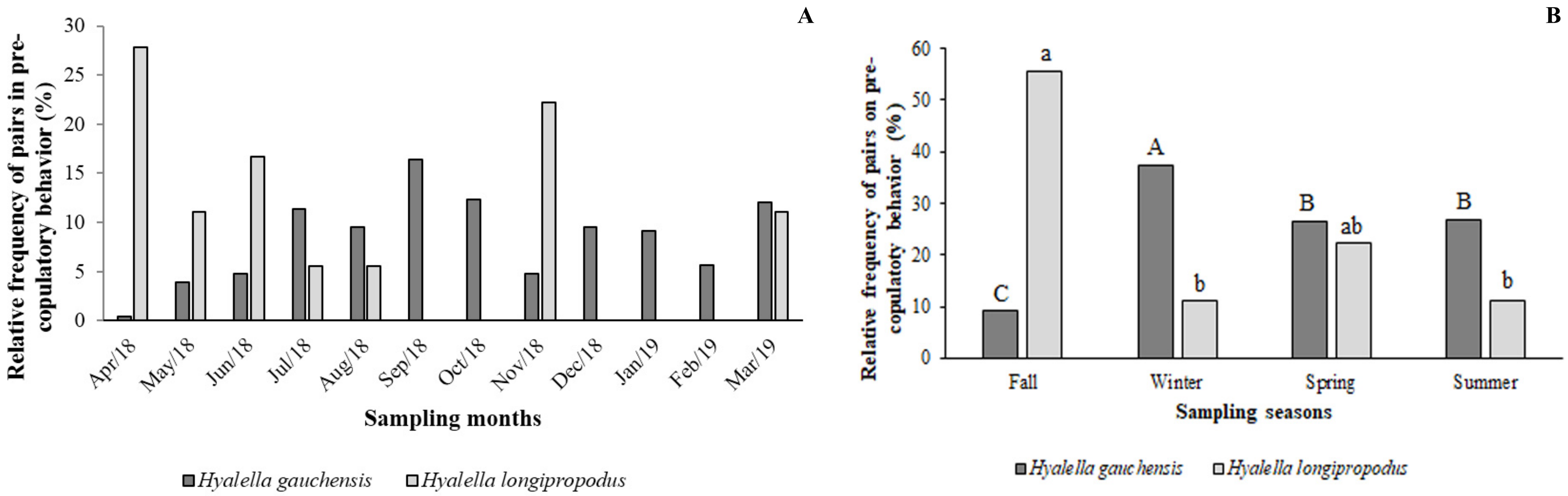

Fig. 8. Monthly (A) and seasonal (B) relative frequency (%) of pairs in pre-copulatory behavior of *Hyalella gauchensis* Streck & Castiglioni, 2017 and *H. longipropodus* Limberger, Graichen & Castiglioni, 2021 northwest region of state of Rio Grande do Sul, Southern Brazil. Different letters above the columns indicate a significant difference (p<0.05) between seasons, where capital letters represent *H. gauchensis* and lower case letters represent *H. longipropodus.*

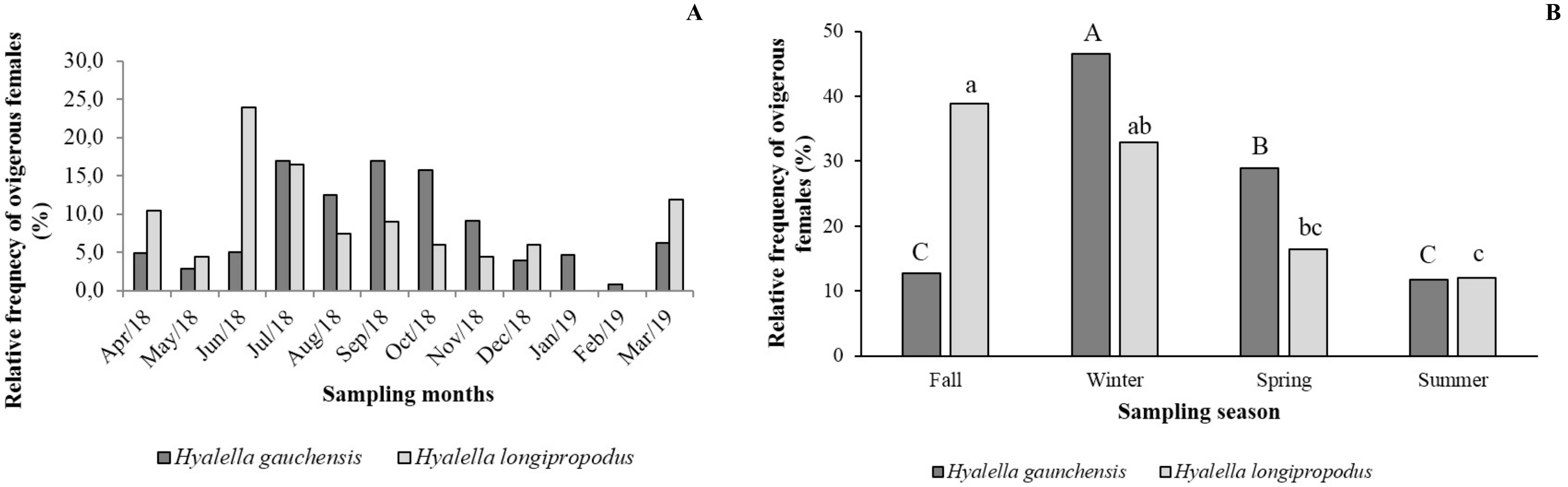

Fig. 9. Monthly (A) and seasonal (B) relative frequency (%) of ovigerous females of *Hyalella gauchensis* Streck & Castiglioni, 2017 and *H. longipropodus* Limberger, Graichen & Castiglioni, 2021 throughout the year in the northwest region of state of Rio Grande do Sul, Southern Brazil. Different letters above the columns indicate a significant difference (p <0.05) between the seasons, capital letters represent *H. gauchensis* and lowercase letters represent *H. longipropodus*.

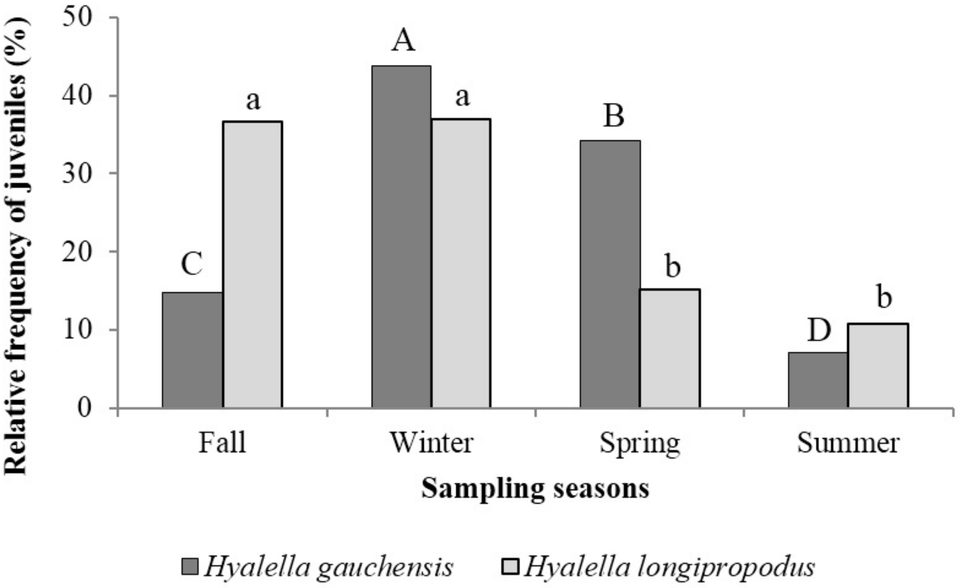

Fig. 10. Frequency of juveniles of *Hyalella gauchensis* Streck & Castiglioni, 2017 and *H. longipropodus* Limberger, Graichen & Castiglioni, 2021 throughout the different seasons of the year in the northwest region of state of Rio Grande do Sul, Southern Brazil. Different letters above the columns indicate a significant difference in the juveniles' frequency ($p<0.05$) among the seasons, where capital letters represent the comparison of *H. gauchensis* and lowercase letters represent *H. longipropodus*.

Both species of *Hyalella* exhibit sexual dimorphism, with males being greater than females and reached sexual maturity at larger sizes (cephalothorax length). This pattern has also been reported in other species, such as *H. pleoacuta* and *H. castroi* (CASTIGLIONI & BOND-BUCKUP, 2008a), *H. bonariensis* (CASTIGLIONI *et al.*, 2016), *H. longistila* (BASTOS-PEREIRA & BUENO, 2016), *H. georginae* and *H. gauchensis* (OZGA *et al.*, 2018), *H. pampeana* Cavalieri, 1968 (COLLA & CÉSAR, 2019), *H. palmeirensis* (CASTIGLIONI *et al.*, 2020), and *H. curvispina* (Shoemaker, 1942) (WALLER *et al.*, 2020). This difference in body size between males and females probably is due to differences in energy allocation. Male amphipod of *H. azteca* allocate more energy towards acquiring females and searching for food, while females invest more energy towards gamete production and parental effort (WEN, 1992).

During the egg incubation period, females do not molt, resulting in a slower growth rate due to the energy expenditure required for embryo production, incubation, and parental care for juveniles within the marsupium (HARTNOLL, 1982; THIEL, 2003). Additionally, larger males have two advantages over smaller males: greater capacity for female acquisition, which enhances transport and maintenance of pre-copulatory behavior, and a better chance of resisting acquisition attempts from other males during this period (WARD, 1983; ADAMS & GREEWOOD, 1993; ADAMS *et al.*, 1985). These features seem to be decisive to explain the body size dimorphism between males and females (HARTNOLL, 1982; WARD, 1983; WEN, 1992; CARDOSO & VELOSO, 1996; DICK & ELWOOD, 1996).

Males and females of *H. longipropodus* are smaller and reach sexual maturity at a smaller body size compared to *H. gauchensis*. This difference may reduce competition between the species as smaller individuals are likely to mature and reproduce earlier than larger individuals, resulting in a higher population size (KRUSCHWITZ, 1978; WELLBORN, 1995). This difference in size at sexual maturity has been previously observed in *Hyalella* species from the Brazilian southern region, for example, in the sympatric species *H. castroi* and *H. pleoacuta* (CASTIGLIONI & BOND-BUCKUP, 2008a), and in non-sympatric species such as *H. gauchensis* and *H. georginae* (OZGA & CASTIGLIONI, 2018). This difference may be due to differences in resource exploration or predation susceptibility.

The size at sexual maturity of female *H. gauchensis* is not consistent between the two methods used to determine it (the size of the smallest female in pre-copulatory behavior and the size of the smallest ovigerous female). This inconsistency has been previously reported for females of *H. pleoacuta* and *H. castroi* by CASTIGLIONI & BOND-BUCKUP (2008a), for *H. gauchensis* and *H. longipropodus* by OZGA & CASTIGLIONI (2017), and for *H. palmeirensis* by CASTIGLIONI *et al.* (2020). These findings highlight the importance of using multiple methods to accurately characterize the size at the onset of sexual maturity in amphipods.

In the present study, the size frequency distribution of cephalothorax length was predominantly bimodal for both species of *Hyalella*, with polymodality observed only in females of *H. gauchensis*. This pattern of bimodality and polymodality is common in *Hyalella* species, as previously observed in *H. castroi* and *H. pleoacuta* (Castiglioni & Bond-Buckup, 2007), *H. longistila* (Bastos-Pereira & Bueno, 2016), *H. bonariensis* (Castiglioni *et al.*, 2016), *H. gauchensis* and *H. georginae* (Ozga & Castiglioni, 2018), *H. pampeana* (Colla & César, 2019), and *H. palmeirensis* (Castiglioni *et al.*, 2020). Additionally, this pattern is observed in several other amphipod species (Cunha *et al.*, 2000; Guerao, 2003; Gonçalves *et al.*, 2003; Marques *et al.*, 2003; Appadoo & Myers, 2004; Subida *et al.*, 2005). Unimodality in size frequency distribution is typically observed in populations with continuous recruitment and constant mortality rates across different life stages, indicating population stability. In contrast, bimodality or polymodality can arise from slow growth in juvenile or adult phases, differential recruitment peaks or migration, mortality, development of sexual maturity, or behavioral differences (Díaz & Conde, 1989). The results of the present study suggest that both species of *Hyalella* have continuous reproduction and recruitment throughout the year, with different reproductive peaks and periods of juvenile entry into the populations. This hypothesis is supported by the presence of pre-copulatory pairs, ovigerous females, and juveniles during all seasons of the year in both populations.

The total sex ratio favored females in both species. This sex ratio pattern has been observed in several populations of *Hyalella* in Brazil, including *H. pleoacuta* and *H. castroi* (Castiglioni & Bond-Buckup, 2008a), *H. bonariensis* (Castiglioni *et al.*, 2016), *H. longistila* (Bastos-Pereira & Bueno, 2016), *H. georginae* and *H. gauchensis* (Ozga *et al.*, 2018), and *H. palmeirensis* (Castiglioni *et al.*, 2020). The desviation in the proportion in favor of females was also observed in a population of *H. curvispina* from the municipality of Montevideo, Uruguay (Waller *et al*., 2020). These deviations in sex ratio are likely related to the reproductive behavior of *Hyalella* species, as males spend more time exposed to the environment while choosing, holding, and carrying females during pre-copulatory behavior, making them more susceptible to predation (Moore, 1981; Powell & Moore, 1991; Wellborn, 1994; Cothran, 2004; Kevrekidis, 2004; Wellborn & Cothran, 2007; Castiglioni *et al.*, 2016).

The seasonal sex ratio favored females in *H. gauchensis* and *H. longipropodus*, except in the summer for *H. longipropodus* when males were more frequent. This predominance of females in most seasons of the year was previously reported by Castiglioni *et al.* (2016) in *H. bonariensis* and also by Castiglioni & Bond-Buckup (2008a) in populations of *H. pleoacuta* and *H. castroi* found in the state of Rio Grande do Sul. However, Ozga & Castiglioni (2018) observed more variations in the sex ratio, with females of *H. georginae* being more frequent only in summer and females of *H. gauchensis* in fall and summer. Usually, in studies with amphipods, males are found in fewer numbers than females throughout the seasons, because some seasonal and structural variations or reproductive behaviors could favor one gender (Wellborn, 1994, 1995; Marsden, 2002; Kevrekidis, 2004; Appadoo & Myers, 2004; Wellborn & Cothran, 2007; Castiglioni & Bond-Buckup, 2008a). This inclination favorable to females can be considered advantageous in population structures, considering that females are responsible for the population's reproductive potential (Cardoso & Veloso, 2001).

Hyalella gauchensis and *H. longipropodus* inhabit a habitat with continuous availability of food and shelter (macrophytes) (personal observation), enabling them to reproduce throughout the year, with greater intensity during periods of lower temperatures. Macrophytes have been observed to provide food and/or shelter for ovigerous females and juveniles, contributing to the species' reproductive success, as noted by Ozga *et al.* (2018). The most intense reproductive activity during colder months may represent a favorable strategy to protect their juveniles from competition and predation by other aquatic invertebrates that inhabit the same ecosystem, as reported by Castiglioni *et al.* (2016, 2020). This reproductive pattern has also been observed in other *Hyalella* species found in Brazil, such as *H. castroi* and *H. pleoacuta* (Castiglioni & Bond-Buckup, 2008a), *H. bonariensis* (Castiglioni *et al.*, 2016), *H. longistila* (Bastos-Pereira & Bueno, 2016), and *H. palmeirensis* (Castiglioni *et al.*, 2020). However, another population of *H. gauchensis* (from the type locality), which does not share the habitat with other species of the same genus, exhibits more intense reproductive activity during the summer (Ozga *et al.*, 2018). The greater abundance of ovigerous females and pre-copulatory behavior in winter was also observed in a population of *H. curvispina* from municipality of Montevideo, Uruguay (Waller *et al*., 2020).

In the present study, a difference was observed in the reproductive peak of the species. *H. longipropodus* seems to anticipate its period of greater reproductive intensity to autumn, followed by winter, while *H. gauchensis* has its reproductive peak in winter followed by spring. This alternation of reproductive peaks has already been observed in other sympatric *Hyalella* species by Castiglioni & Bond-Buckup (2008a), with *H. pleacuta* reproduces with greater intensity in winter, while *H. castroi* had its reproductive peak in autumn. This reproductive strategy may temporarily separate the incidence of a large number of juveniles of each species, thereby reducing the competition for resources among these individuals. As a result, it provides a greater probability of success in the survival of their offspring.

Although *H. gauchensis* and *H. longipropodus* exhibit continuous reproduction and recruitment, it was observed that each species had a specific season for their peak in reproduction and recruitment (autumn for *H. longipropodus* and winter for *H. gauchensis*). These results differ from previous observations of *H. gauchensis* in the type locality, which showed seasonal differences between the peaks of reproduction (summer) and recruitment (spring) (Ozga *et al.*, 2018). In *H. palmeirensis*, ovigerous females and pre-copulatory pairs were found throughout the year, with high frequency in winter and autumn, respectively, and juveniles were sampled throughout the year, with greater intensity in spring (Castiglioni *et al.*, 2020). Considering the duration of the embryonic period and parental care, females are capable of becoming ovigerous and releasing offspring within the same season (Castiglioni & Bond-Buckup, 2007; Geisler, 1944; Cooper, 1965).

CONCLUSION

The sympatric species *H. gauchensis* and *H. longipropodus* showed some distinct population characteristics. Among them, their reproductive strategies can be highlighted, where the two species showed their peak of reproduction and recruitment in the coldest periods of the year. However, the seasonal temporal separation of these behaviors between the two populations may indicate the different evolutionary adaptations between the two species to enable their coexistence.

According to the analysis, both *H. gauchensis* and *H. longipropodus* demonstrate stability in their population structure, with reproductive activity and a representative presence of males, females and juveniles throughout the year. However, *H. longipropodus* showed lower abundance, small body size, small body size onset sexual matutiry when compared to *H. gauchensis*. Probably, *H. gauchensis* is a superior competitor that can allocate more resources in the shared habitat, and then growth and reproduce more than *H. longipropodus*. This last eventually may become sexually mature earlier trying to overcome such competition, reproducing faster and exploring other niches. However, further studies could clarify these questions or support a greater understanding of this population characteristic in this location.

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