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ECOSYSTEMS

Diversity, phenology and voltinism of Chironomidae (Diptera). Neotropical streams as a study model

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Abstract: Studies of the relationships between the temporal variation of biological phenomena and environmental factors are essential to understanding the dynamics of communities. Phenology is a structuring element and, together with voltinism, is related to timing of reproductive activities. The aim of this study is to ascertain the structure and composition of the Chironomid assemblage and its emergence patterns in relation to the environmental variables through the use of the Chironomid pupal exuviae technique using the Neotropical streams as a model. The streams presented similar species compositions. The highest density of pupal exuviae was recorded during spring and the greatest richness in late summer and early autumn. Chironominae presented greater prevalence throughout the year with oscillations for Orthocladiinae and Tanypodinae. Chironominae and Tanypodinae presented emergence peaks in spring-summer and Orthocladiinae in autumn-winter. Rainfall, photoperiod and water and air temperatures structured the Chironomid assemblage, influencing emergence. Univoltine, bivoltine and multivoltine species were recorded. The species presented interspecific synchronization in emergence peaks at different times of the year.

Key words: Chironomid Pupal Exuviae Technique (CPET), assemblage dynamics, emergence patterns, seasonal synchronisation, Neotropical Region.

INTRODUCTION

The family Chironomidae is the most highly represented group of insects in freshwater ecosystems (Cranston 1995). The number of chironomid species generally represents around 50% of the macroinvertebrate composition in these environments (Ferrington et al. 2008).

The main factors that influence Chironomidae abundance are temperature and other environmental parameters such as food supply and presence of competitors and/or predators. Several Chironomidae species are generalists, though they have also conquered and colonised a great variety of rather specialised niches (Spies et al. 2009).

Studies of the relationships between temporal variation of biological and environmental factors, especially climate, are essential to understanding community dynamics (Scott & Epstein 1987). Survival, development and reproduction must be synchronised with the favourable seasons and the diapause with unfavourable periods (Tokeshi 1995). An important aspect of the life cycle is the timing of reproductive activities, in which voltinism and phenology are very important factors (Tokeshi 1995). Voltinism specifies the frequency of annual reproduction and phenology refers to the actual seasonal time of reproduction. In the context of global climatic change, phenological studies have received greater attention in conservation biology, since there is evidence that climatic fluctuations affect ecological processes (Stenseth et al. 2002, Edwards & Richardson 2004, Durance & Ormerod 2007, Domisch et al. 2011, Woodward et al. 2016). Phenology is therefore an important structuring element in almost all areas of ecology and evolution (Forrest & Miller-Rushing 2010). As a group, Chironomidae show a wide variation in phenology, with emergence being observed practically all year round (Pinder 1974).

The collection of pupal exuviae provides accurate measurement of the successful use of a habitat by the immature stages, representing the final point of the aquatic phase. The *Chironomid Pupal Exuviae Technique*, CPET, is a simple, fast technique applicable both to deep rivers with greater flow and to small, shallow streams (Lindegaard 1995). CPET samples provide material to analyse different ecological characteristics and it enables faster and more accurate identification than when larvae are considered. The chironomid assemblage can thus be presented in an integrated manner (Raunio et al. 2007a, b, Kranzfelder et al. 2015) and it is possible to obtain a “fingerprint” from a lotic environment from the results obtained through CPET.

In the northern hemisphere, CPET has been used for monitoring fluvial environments for several decades (Raunio & Muotka 2005, Kranzfelder et al. 2015), as it is associated to the physicochemical variables of the environments. In the southern hemisphere, CPET has been applied for the study of spatial and temporal variation in lotic environments in southern Australia (Hardwick et al. 1995), southern Brazil (Siqueira et al. 2008), and north-eastern and southern Argentina (García & Añón-Suárez 2007, Mestre et al. 2018).

The aim of the present study is to ascertain the composition and structure of the Chironomidae assemblage and its emergence patterns in relation to the environmental variables by using the Chironomidae Pupal Exuviae Technique (CPET), using the streams of the Pampean plains as models.

To do so, we developed a field design, during which the following questions arose: (i) Is there any relationship between Chironomidae emergence and environmental variables? (ii) Are there any patterns in phenology and voltinism in the Pampean species throughout the year?

MATERIALS AND METHODS

Study area

The study area of the present study comprises the southern sector of the Argentine Pampean grasslands. Rainfall and underground water feed into the streams, which flow at low current velocity over a relief of gently sloping plains (0.3 m.km^{-1}). The streambeds present a hard, homogeneous substratum, with high calcium carbonate content on which thin sediments are usually deposited (mainly silt and clay), with total lack of rocks or pebbles. A wide range of macrophytes grows on the streambeds, though their presence is variable and their coverage heterogeneous, depending, among other factors, on the season and the occurrence of heavy rainfall. The lack of autochthonous riverside forest determines high irradiation, even in the upper reaches of the streams, while the low current velocity and high nutrient level in the Pampean water bodies lead to regular eutrophication (Giorgi et al. 2005, Feijoó & Lombardo 2007). According to Köppen’s classification, the climate in the study sector is temperate with oceanic influence. Summers are warm and winters are cool and variable, with frequent frosts, but without snow. Average annual temperature is 17 °C, with high humidity in all seasons. The region’s wide thermal variation causes very warm days during winter and, less frequently, days with low temperatures in summer. Rainfall is concentrated in the spring and summer months (source: Argentine National Weather Services).

Due to the high level of river and stream modification in the Pampean grasslands, it is impossible to find sites without alteration in land use, relatively undisturbed streams were therefore selected. This means that although the selected streams are not pristine, they have low levels of perturbation and therefore natural variations in morphological, physical and chemical characteristics. The selected sites are: El Pescado Stream (PES) (34°57’36.6”S-57°46’36.6”W), Cajaravilla Stream (CAJ) (35°02’25.3”S-57°48’54.8”W), Tubichamini Stream (TUB) (35°07’22.3”S-57°41’11.7”W), Buñirigo Stream (BUN) (35°08’38.1”S-57°34’12.6”W) and Juan Blanco Stream (JB) (35°08’29.7”S-57°26’27.8”W), all of them mainly influenced by agricultural and livestock activity (Fig. 1).

Field design

Sampling was carried out during an annual cycle from October 2016 to September 2017 (due to heavy rainfall prior to some of the sampling dates, which made the sites impossible to access, samples were not taken in January and July and only three of the five sites were sampled in February). To collect pupal exuviae, we used a D-net 25 cm in diameter and 250 µm mesh opening. We collected Chironomidae pupal exuviae by active sampling because of the slow current in the streams, with 3 samples from each site, in an area of 30 m^2 (30 m x 1 m) as sampling effort unit at each site. The field samples were fixed *in situ* with 80% Ethanol. The pupal exuviae were separated under stereoscopic microscope at 10X magnification and preserved in 80% Ethanol. We prepared permanent

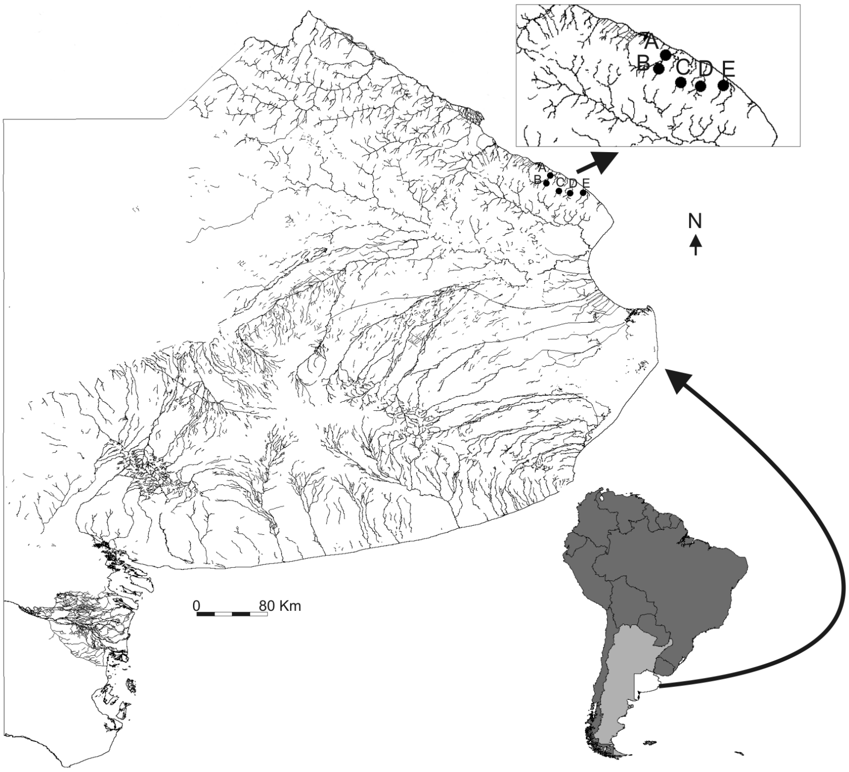

Figure 1. Map of Pampean stream in study. A: El Pescado Stream; B: Cajaravilla Stream; C: Tubichamini Stream; D: Buñirigo Stream; E: Juan Blanco Stream.

microscope slides with pupal exuviae material following the technique proposed by Pinder (1986), for subsequent taxonomic identification to the lowest possible level, using specific literature for the region and the corresponding keys (Wiederholm 1986, Wiedenbrug 2000, Wiedenbrug & Ospina-Torres 2005, Merrit et al. 2008, Prat et al. 2014, Paggi 2009, among others). The specimens are deposited in the collection of Instituto de Limnología "Dr. Raúl A. Ringuelet" (ILPLA-CONICET-UNLP).

Most of the species and some of the genera represent taxa for which the pupal condition is unknown. As a result, many taxa were considered "morphospecies" (e.g. *Ablabesmyia* sp.1) or unknown genera (e.g. undetermined Chironomini 1). Hereinafter, all taxa determined in this study will be referred to as "species".

Along with the collection of exuviae, we measured the following physicochemical parameters in the field: current velocity (m/s) measured mid-channel (average of three measurements) by timing a float as it moved over a known distance (Gordon et al. 1994), temperature and dissolved oxygen (DO) (dissolved oxygen meter Lutron YK-22DO), pH (Universal pH Test Paper Strips), conductivity (AD204 Standard conductivity pocket tester) and transparency (Secchi disk). We took water samples for laboratory determination of nitrate ($N\text{-}NO_3$), nitrite ($N\text{-}NO_2$), ammonium ($N\text{-}NH_4$), total phosphorous ($P\text{-}PO_4$), biological oxygen demand (BOD_5) and chemical oxygen demand (COD), following standard methods (APHA 1998). At each sampling session, we weighted the percentage of macrophyte coverage by means of the Mapping technique (Feijoó & Menéndez 2009). We also included other variables such as total rainfall (mm), mean air temperature (°C) and photoperiod (mean hours of light) provided by La Plata Observatory Meteorological Station which is placed close to the study area (34°54'24"S-57°55'56"W, 15 m a.s.l) (School of Astronomical and Geophysical Sciences, UNLP, unpublished data).

Data analysis

Assemblage analysis

The following assemblage metrics were calculated from the data: exuviae density (number of exuviae/m^{-2}), taxonomic richness (S), Shannon-Wiener diversity index (H´) and equitability index (J') (H'/Hmax), through the PAST 3.0 statistical program (Hammer et al. 2001).

We analysed all the environmental variables to detect differences among sites using ANOVA and, when the variables did not meet the assumptions of normality and variance, we used homoscedasticity, Kruskal-Wallis non-parametric tests, using the R-language available in the Agricolae library (de Mendiburu 2019).

Phenological analysis and voltinism

We analysed the emergence patterns of Chironomid assemblages. For each species, we determined the months in which emergence was recorded. In order to determine the voltinism of the study species, we used XY-plot, where average exuviae density was plotted for each sampling month. We used the number of emergence peaks (months when the highest number of collected exuviae was recorded) to obtain an approximation to knowledge of voltinism in Pampean species. The species that presented a single annual peak of emergence are considered univoltine, the species presenting two emergence peaks separated by two or more months without records are considered bivoltine, and the species appearing throughout most of the year are considered multivoltine. In addition, we used the Kernel density estimation with the pre-determined function "kdensity" in the statistical software R (Moss & Tveten

2019). The Kernel density estimation is a non-parametric way of estimating the likelihood density function of a random variable, in this case, the emergence of pupal exuviae over time. We used this method to exclude any potential sampling error, which could occur, for instance, due to the low likelihood of finding a species as a result of its low abundance. Given a record sample of pupal exuviae density of a species, the estimate of Kernel density extrapolates the data and predicts the emergence period and the relative occurrence of individuals in the entire population during the year under study. Univoltine species will have unimodal Kernel density distribution, bivoltine species will have bimodal distribution and multivoltine species will have multimodal distribution. By analysing the mode number and the temporal variation, we determined voltinism for each species (available from the Author).

In addition, we carried out a Spearman correlation between the metrics of each tribe and the physicochemical and environmental variables to determine which factors affected emergence significantly ($p < 0.05$). These analyses were carried out using the R-language available in the Hmisc library (Harrell & Dupont 2019).

RESULTS

Assemblage analysis

A total 52,057 exuviae were recorded for the different sites, representing a total 36 genera and 62 species, belonging to three subfamilies. The chironomid composition consisted of 17 Chironominae genera, 10 Tanypodinae and nine Orthocladiinae. Of the total species, 34 belonged to the subfamily Chironominae, 14 to Tanypodinae and 13 to Orthocladiinae (Table I). Total richness of the species collected at each site and date ranged from 1 to 33. Density, in general, ranged from 0.01 to 156.53 exuviae m^{-2}. The prevailing taxa, with abundance higher than 10%, were *Parachironomus* sp.2, *Polypedilum* sp.A and *Rheotanytarsus* sp.1, and collectively these species represented 53% of total collected exuviae. Physicochemical variables were within the ranges usually recorded for Pampean streams (Table II). Average depth of the streams under study was 78 cm. Mean current velocity was highest in BUN (0.53 $cm.seg^{-1}$) and lowest in JB (undetectable). Maximum transparency was observed in BUN and CAJ, with values of about 30 and 40 cm, respectively. Mean percentage of macrophyte cover was 46%, with maximum values up to 95% (CAJ). Mean conductivity was 0.88 mS, with the highest values (1.61 mS) recorded in CAJ. The pH values ranged from 7.09 to 7.60. Mean DO was 6.46 mg/l, with maximum values of 8.2 mg/l in BUN. CAJ presented the highest values of the different nutrients. BOD_5 and COD were lowest in BUN. Tables III and IV show the results of the ANOVA and Kruskal-Wallis tests. The variables that presented significant differences among the analysed sites were P-PO_4, current velocity, macrophyte coverage, DO, conductivity, depth and transparency.

Spearman's correlation (Supplementary Material - Table SI) showed that rainfall, photoperiod and water and air temperature correlated positively with the different ecological metrics of Chironominae and Tanypodinae, while Orthocladiinae correlated negatively with air temperature. There was a positive correlation of DO with Chironominae and Orthocladiinae. The three subfamilies correlated negatively with conductivity, BOD_5, COD, nutrients, macrophyte coverage and current velocity.

Table I. **List of Chironomidae (Diptera) collected in pampean streams. The emergence period, emergence peak (Δ) and Voltinism are shown.**

	Spring			**Summer**		**Autumn**			**Winter**		**Voltinism**
TAXA	**O**	**N**	**D**	**F**	**M**	**A**	**M**	**J**	**A**	**S**	
CHIRONOMINAE											
Chironomini											
Apedilum elachistus				x	x	Δ					Univoltine species with its optimum in autumn.
Axarus sp.	x		x	Δ	x	x	x	x	x	Δ	Univoltine species, with diapause in winter.
Chironomus calligraphus	x				x	Δ	x	x	x	x	Univoltine species, with its optimum in autumn.
Cryptochironomus sp.1			x	Δ		Δ	x		x		Multivoltine species with emergencies from late spring to autumn and diapause in winter.
Cryptochironomus sp.2			x		x	Δ	x		x	Δ	Multivoltine species with its optimum in autumn.
Dicrotendipes embalsensis					x	Δ					Univoltine species with its optimum in autumn.
Dicrotendipes nestori	Δ	x	x	x	x	x	x		x	x	Multivoltine species with diapause in winter.
Einfeldia sp.	Δ	x	x	x	x	x	x	x	x	x	Univoltine or facultative bivoltine specie. It was presented emergency peak during distinct seasons in the different streams.
Goeldichironomus maculatus		Δ			Δ						Univoltine species, with emergencies in spring and late summer.
Goeldichironomus sp.1				Δ	x	x					Univoltine species with its optimum in summer.
Parachironomus longistilus	x	x	x	x	x	Δ	x	x	Δ	x	Univoltine or facultative bivoltine species with emergence peaks during different seasons.
Parachironomus sp.2	Δ	Δ	x	Δ	x	x	x	x	Δ	x	Multivoltine species with highest density all year long with exception in autumn.
Paralauterbormiela sp.	x	x	x		Δ	x	x			x	Bivoltine species with diapause in summer.
Polypedilum sp.A	Δ	x	x	x	x	x	x			x	Univoltine or facultative bivoltine species with optimum in spring and relatively low density values during the rest of the year (< 2 ind.m^{-2}).

Table I. Continuation.

Polypedilum sp.B	Δ	x	x		x	x	x	x	x	x	Bivoltine or facultative multivoltine species with its optimum in spring and relatively low densities values during the rest of the year.
Polypedilum sp.C			Δ								Univoltine species with emergence peak in early summer.
Polypedilum sp.D					Δ	x					Univoltine species with emergencies in late summer and early autumn.
Polypedilum sp.E										Δ	Univoltine species with emergence peak in late winter.
Polypedilum sp.F								Δ			Univoltine species with its optimum in late autumn.
Polypedilum sp.G		x	x				x	x	x	Δ	Univoltine species with diapause in summer autumn.
Unknown Chironomini #2	x	x	x		Δ	x	x	x	Δ	x	Multivoltine species with its optimum in late summer and suboptimum in late winter.
Unknown Chironomini #3		Δ		x							Univoltine specie with its optimum in spring and a small emergence peak during summer.
Pseudochironomini											
Aedokritus nr *sartori*					Δ						Univoltine species with emergence peak in summer.
Pseudochironomus sp.	Δ	x				Δ			Δ	Δ	Bivoltine species with its optimum in winter and spring and emergencies in April (autumn).
Pseudochironomus viridis									Δ		Univoltine species with emergence peak in winter.
Tanytarsini											
Paratanytarsus corbi	x				x	Δ					Bivoltine species with its optimum in late summer and early autumn.
Paratanytarsus grimmi	Δ	Δ	x			x	x	x	x	x	Univoltine or facultative bivoltine species with its optimum in spring.
Rheotanytarsus sp.1	Δ	Δ	x	x	x	x	x	x	x	x	Multivoltine species with its optimum in spring and late autumn (Buñirigo stream). Relatively low-density values were recorded during the rest of the year.
Rheotanytarsus sp.2		Δ									Univoltine species with its optimum in spring.

Table I. Continuation.

Tanytarsus sp.1	Δ	x	x	x	x	x	x	x	Δ	x	Multivoltine species with emergencies all year long and optimum in late winter - early spring.
Tanytarsus sp.2		x		Δ	x	x					Univoltine or facultative bivoltine species with its optimum in summer and suboptimum in autumn.
Tanytarsus sp.3				Δ	x	Δ				x	Univoltine or facultative bivoltine species with its optimum in summer.
Tanytarsus sp.4					Δ	Δ		x	Δ		Bivoltine species with diapause in warm months and optimum in autumn.
Tanytarsus sp.5					x	Δ					Univoltine species with its optimum in autumn.
ORTHOCLADIINAE											
Corynoneurini											
Corynoneura sp.	Δ	x									Univoltine species with its optimum in spring.
Lopescladius sp.					Δ				x	x	Univoltine species with its optimum in summer.
Onconeura analiae			Δ								Univoltine species with its optimum in late spring.
Thienemanniella liae	x	x	x	x	Δ	x	x	x	x	Δ	Univoltine or facultative bivoltine species with emergencies all year long.
Thienemanniella sp.2	x	Δ	x				x				Univoltine species with its optimum in spring.
Metriocnemini											
Allocladius neobilobulatus	x	Δ	x								Univoltine species with its optimum in spring.
Limnophyes natalensis	Δ										Univoltine species with its optimum in spring.
Pseudosmittia sp.	Δ	Δ							Δ		Univoltine species with diapause in summer-autumn.
Unknown Metriocnemini sp.1	x			x			x	x	Δ	x	Univoltine or facultative bivoltine species with its optimum in winter.
Orthocladiini											
Cricotopus sp.1	Δ	x						x	Δ		Univoltine species with its optimum in spring and winter.
Cricotopus sp.2	Δ	x	x	x	x	x	x	x	x	x	Univoltine or facultative bivoltine species with its optimum in spring and emergencies all year long at very low densities.
Cricotopus sp.3									x	Δ	Univoltine species with its optimum in late winter.

Table I. Continuation.

Nanocladius sp.	x	Δ	x		x				x	x	Univoltine or facultative bivoltine species with emergency peaks in spring.
TANYPODINAE											
Clinotanypodini											
Coelotanypus sp.	Δ	x	x	x	x	x					Bivoltine species with diapause in winter.
Pentaneurini											
Ablabesmyia nr *fusariae*	x	x	x	x	x	Δ	x				Univoltine species with diapause in winter.
Ablabesmyia sp.1	x	x	x	x	x	Δ	x	x		x	Multivoltine species with its optimum in autumn and emergencies all year long but at low densities.
Ablabesmyia sp.2				x	Δ				x	x	Bivoltine species with emergencies in summer and winter.
Labrundinia nr *fiorelinii*						Δ		x			Univoltine species with its optimum in autumn.
Labrundinia sp.1		x			x	Δ	x				Univoltine or facultative bivoltine species with its optimum in autumn.
Labrundinia sp.2		x	x		Δ	x	x	x	x	x	Univoltine or facultative bivoltine species with its optimum in late summer.
Larsia sp.1		x			x	Δ					Bivoltine species with diapause in winter.
Larsia sp.2						Δ				x	Univoltine species with emergencies in autumn and late winter.
Metapelopia sp.			Δ	x	x				x	Δ	Bivoltine species with its optimum in late winter .
Monopelopia nr *paranaense*			x	x	Δ	x	x				Univoltine species with its optimum in late summer and early autumn.
Procladiini											
Djalmabatista sp.					Δ					Δ	Univoltine species with diapause in spring and autumn.
Procladius sp.					Δ						Univoltine species with its optimum in late summer.
Tanypodini											
Tanypus nr *urszulae*			Δ	Δ							Univoltine species with its optimum in summer.
Tanypus sp.		x	Δ				x	x	Δ	x	Bivoltine species with its optimum in spring, diapause in summer and low emergencies in winter.

Table II. Mean values (min-max) of the physicochemical parameters recorded at the sampling sites.

	A° Buñirigo	**A° Cajaravilla**	**A° Juan Blanco**	**A° El Pescado**	**A° Tubichamini**
WT (°C)	**20.02** (15.30-26.80)	**17.88** (14.10-27.50)	**21.04** (14.50-29.40)	**20.76** (13.40-26.50)	**19.52** (12.60-30.80)
Depth (cm)	**78.15** (64.67-103.00)	**73.35** (48.67-99.33)	**90.98** (73.67-105.00)	**72.41** (47.00-88.33)	**75.43** (60.67-90.67)
VEL (cm.seg^{-1})	**0.53** (0.00-4.33)	**0.30** (0.10-0.86)	**0.00** (0.00-0.03)	**0.28** (0.03-0.51)	**0.07** (0.00-0.25)
TRANSP (cm)	**39.37** (21.00-70.00)	**30.83** (19.00-49.00)	**14.89** (6.00-22.00)	**8.42** (5.00-18.00)	**18.27** (9.00-32.00)
MACROP (%)	**22.78** (10.00-45.00)	**94.44** (80.00-100.00)	**51.39** (20.00-77.50)	**7.78** (5.00-10.00)	**54.50** (15.00-80.00)
COND (mS)	**0.67** (0.32-0.97)	**1.61** (0.49-8.20)	**0.40** (0.15-0.66)	**0.92** (0.48-1.25)	**0.79** (0.42-1.04)
pH	**7.6** (6.2-8.9)	**7.09** (6.8-7.5)	**7.35** (6-8.8)	**7.09** (6.5-7.5)	**7.21** (7.0-7.9)
DO (mg/l)	**8.195** (5.467-10.500)	**4.379** (2.600-9.867)	**5.990** (3.600-11.167)	**6.805** (3.500-12.200)	**6.931** (4.300-12.000)
P-PO_4 (mg P/l)	**0.096** (0.011-0.453)	**0.321** (0.060-0.690)	**0.101** (0.019-0.224)	**0.295** (0.065-0.469)	**0.110** (0.014-0.256
N-NO_3 (mg N/l)	**0.078** (0.026-0.141)	**0.285** (0.055-0.986)	**0.107** (0.023-0.208)	**0.100** (0.019-0.243)	**0.053** (0.016-0.139)
N-NO_2 (mg N/l)	**0.005** (0.001-0.015)	**0.031** (0.001-0.077)	**0.010** (0.004-0.035)	**0.016** (0.002-0.057)	**0.013** (0.001-0.037)
N-NH_4 (mg N/l)	**0.028** (0.001-0.137)	**0.122** (0.001-0.375)	**0.051** (0.001-0.280)	**0.091** (0.001-0.361)	**0.015** (0.001-0.060)
BOD_5 (mg O_2/l)	**10** (5-16)	**12** (6-23)	**21** (3-35)	**19** (9-33)	**25** (6-118)
COD (mg O_2/l)	**28** (9-70)	**39** (10-73)	**60** (7-95)	**57** (14-88)	**63** (12-168)

Table III. One-way ANOVA test on the physicochemical, hydrological and environmental variables recorded at the sampling sites. In bold type, the significant p values ($p \leq 0.05$) and the corresponding post-hoc test.

Source of variation	***gl***	***F***	***p- value***	**post-hoc test**
COD	4	2.69	**0.0434**	CAJ < JB, PES, TUB; BUN < PES
COND	4	3.35	**0.0180**	JB < CAJ, PES, TUB
DEPTH	4	2.70	0.0433	CAJ, PES, TUB < JB
TRANSP	4	17.26	**0.0000**	PES < JB, TUB < BUN, CAJ
DO	4	0.36	0.8351	no significant differences
PHOT	4	0.02	0.9989	no significant differences
RAINF	4	0.18	0.9475	no significant differences
AT°	4	0.05	0.9946	no significant differences
WT°	4	0.50	0.7334	no significant differences

Table IV. Kruskal-Wallis non parametric test on the physicochemical and hydrological variables recorded at the sampling sites. In bold type, the significant p values ($p \leq 0.05$) and the corresponding post-hoc test.

Source of variation	*H*	*p- value*	Test posteriori
P-PO_4	14.7059	**0.00535**	BUN < PES
VEL	25.9814	**0.00003**	JB < CAJ, PES
MACROP	39.4075	**5.7×10^{-8}**	PES < CAJ, JB, TUB; BUN < CAJ
pH	6.45686	0.16753	no significant differences
N-NO_3	5.72896	0.22032	no significant differences
N-NO_2	5.83279	0.21199	no significant differences
N-NH_4	4.10802	0.39158	no significant differences
BOD_5	9.16835	0.05703	no significant differences

Phenological analysis and voltinism

The highest density of pupal exuviae was recorded during spring (October and November), whereas the lowest values were observed in February, May and June (Figure 2). As regards richness, the number of chironomid species that emerged was higher in late summer and early autumn (March and April). The lowest richness values agreed with the months when lower densities were found. Overall, we observed prevalence of the subfamily Chironominae throughout the annual cycle and oscillations for the other two subfamilies. Orthocladiinae prevailed in spring and late summer, while Tanypodinae prevailed in early autumn.

Analysis of Chironomidae phenology revealed the emergence periods of the different species under study. Chironominae presented maximum emergence peaks in October (237 exuviae.m^{-2}) and November (104 exuviae.m^{-2}); Orthocladiinae in October (15 exuviae.m^{-2}) and November (13 exuviae.m^{-2}) and Tanypodinae in April (9 exuviae.m^{-2}). During the rest of the year, the values for Chironominae did not exceed 35 exuviae.m^{-2}, whereas Orthocladiinae and Tanypodinae did not exceed 2 exuviae.m^{-2} (Figure 3). As regards specific richness, Chironominae presented the maximum values in March and April (24 and 25 species, respectively), Tanypodinae in March (11 species) and Orthocladiinae in October and November (8 and 10 species, respectively). The comparison of exuviae density with taxa richness shows that in March and April, the highest number of species in emergence and highest densities were recorded in the subfamily Orthocladiinae and Tanypodinae. On the other hand, in October, Chironominae presented the highest emergence density, but the lowest number of species.

Differences in the emergency peaks of the analyzed species (n=62) resulted in a statistical significant variation in the emergence patterns throughout the year (Table I). Analysis of the life cycles of the species identified in our study area showed that most species are univoltine (41 species), while fewer are bivoltine or multivoltine (12 and 9 species, respectively). Among the univoltine species, we identified 10 species with facultative bivoltinism. Among the bivoltine species, two are facultative multivoltine (Table I). Table SII shows the criteria used to classify type of voltinism, through XYplot and Kernel density.

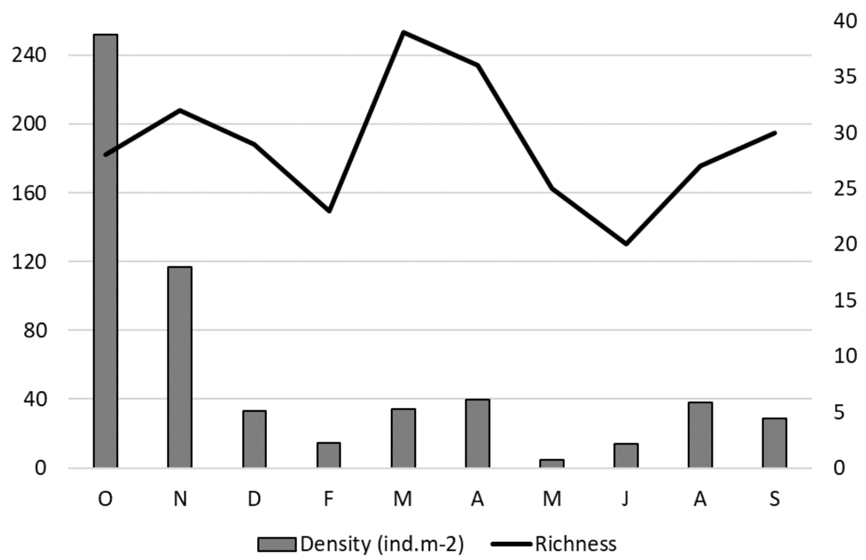

Figure 2. **Monthly variation of the density and richness of the Chironomidae assembly present in the study streams.**

DISCUSSION

Assemblage analysis

When thorough sampling is performed, Chironomidae species richness is often found to be among the highest for aquatic insect families (Ferrington 2008). Coffman (1989) showed that stream order plays a very important role in determining the species richness of the assemblages. Generally, the streams of higher order have more species than currents of lower order, probably as a result of several factors that affect physical and biological heterogeneity differently. Coffman mentions that mean species richness is 26 (10-64) for first order streams, 44 (13-144) for second order streams and 63 (25–157) for third order streams. In the present study, 62 species were found, which is similar to the maximum number reported by Coffman (1989) for streams of first order

Chironomidae Phenology

Following the classification by Ashe et al. (1987) the Neotropical studies by Siqueira et al. (2008), Mestre et al. (2018) and the present study are all representative of the tropical-subtropical fauna, whereas the study by García and Añón-Suárez (2007) represents Andean-Patagonian fauna. The Pampean streams under study were dominated by Chironominae species. According to Pinder (1995), the species included in Chironominae are adapted to living in soft sediments and able to tolerate the higher temperatures, and occasionally the lower oxygen concentrations that occur in the large rivers of lowlands, such as those characteristic of the region under study. However, in environments with higher current velocity, or where there is aquatic vegetation, Orthocladiinae and other taxa characteristic of rhithron can proliferate

Ashe et al. (1987) suggest, as general rule, that from the polar regions to the Equator (i.e., high to low latitudes) or from high mountains to lowlands, the diversity and abundance of Chironominae and Tanypodinae increase with a corresponding decrease in Orthocladiinae, Podonominae, Diamesinae and Prodiamesinae. This rule reflects the adaptations of the main subfamilies to prevailing environmental conditions (oxygen concentration, water temperature, climate, altitude, current velocity, etc.).

The sampling records for one year revealed fluctuations in the emergence of the most

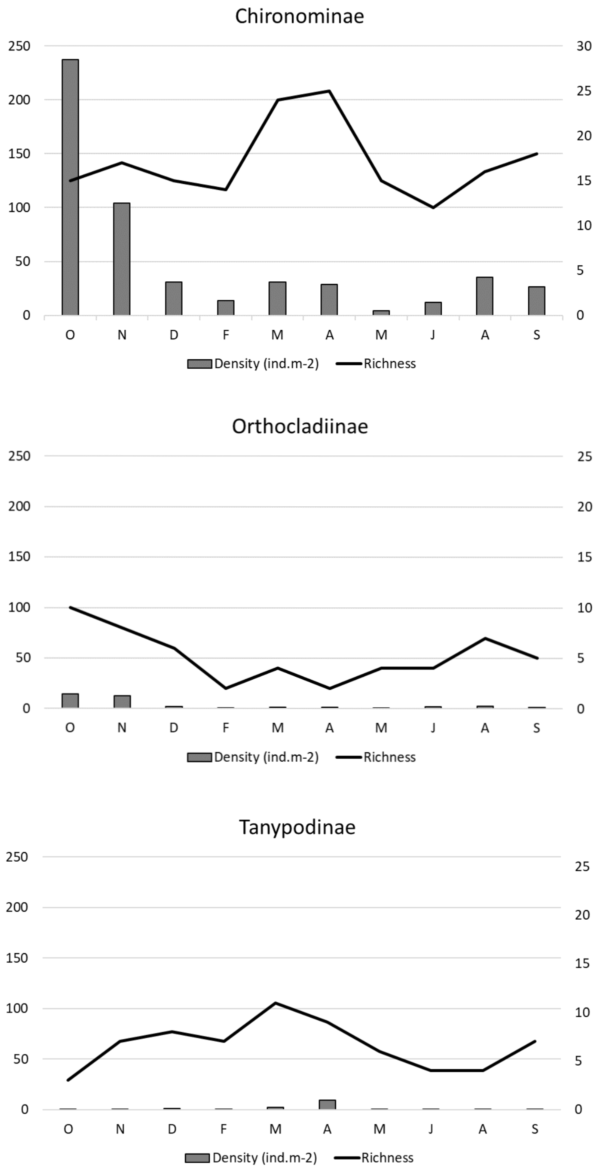

Figure 3. Monthly variation in the density and richness of the Chironomidae subfamilies.

frequent species. Following Coffman (1973), voltinism in Chironomidae can be determined by using the annual record of species emergence. Therefore, the main peaks can reflect the number of generations of each species. In the present study, there was a prevalence of univoltine, bivoltine and multivoltine respectively. Orthocladiinae species presented univoltine cycles while Chironominae species were mainly univoltine or multivoltine. Tanypodinae species presented similar proportions in univoltine and bivoltine cycles. In lentic environments with higher temperatures the prevailing Chironominae and Tanypodinae generally have multivoltine life cycles with higher numbers of generations (Zilli & Paggi 2013). In cold temperate environments, insect emergence is synchronised to the short summer period (Butler 1982) with mainly univoltine cycles, while in warm environments, emergence periods are longer (Tokeshi 1995, Stagliano et al. 1998), with prevalence of multivoltine cycles and generation overlap (Benke & Jacobi 1994). The higher proportions of univoltine species may be due to the fact that temperate climate predominates in the study area, with very high temperatures during warm seasons and very low temperatures in winter, creating a wide temperature range over the year. Because insects are ectothermic organisms, they are strongly affected by environmental temperature, rainfall, and photoperiod (Valtonen et al. 2013, Bonal et al. 2015). In this way, changes in phenology have been one of the most obvious signs of global warming (Forrest 2016). Furthermore, in many insects that complete a variable number of generations in a year, warming can affect generation time. Longer and warmer seasons are allowing many insect populations to complete more generations per year than used to be possible in the past. Although more studies are still required, CPET could be used to observe possible changes in the life cycles of species as a consequence change in the environment, since an increase in temperature would support the development of multivoltine species at the expense of bi and univoltin species (Altermatt 2010, Pöyry et al. 2011).

Despite the short distances separating the streams, the emergence peaks of some of the univoltine species occurred on different streams at different seasons. This could be related with differences of some environmental variables between streams such as COD, conductivity, depth, transparency, P-PO4, current velocity and percentage of macrophyte coverage, and its influence on the emergences of the adult stage. The term facultative voltinism is introduced in this study to describe an overlap of curves in the graphs. This may be due either to generation overlap (asynchronous generations) or environmental changes providing favourable conditions for longer, recording other emergence peaks with lower densities. In the study area, there are often warm days during winter, which may enable emergence when the climatic conditions are within the tolerance range of the species.

Some species recorded in this study presented coinciding emergence peaks at different times of the year, indicating interspecific emergence synchrony. The species *Cryptochironomus* sp.1 and *Tanytarsus* sp.3 presented emergence peaks in summer (February) and autumn (April). The species *Thienemanniella liae*, *Ablabesmyia* sp.2, and *Djalmabatista* sp. registered synchrony in summer and winter (March, September). Finally, the species *Cricotopus* sp.1 and *Tanytarsus* sp.1 presented emergence peaks in spring (October) and winter (August). In each species, both annual peaks had similar densities. These species belong to different subfamilies, which means that there was no temporal sequence, as has been proposed for temperate streams (Coffman 1973).

The development and emergence time in chironomids are determined by environmental conditions and the species-specific responses to them. In the present study, the Chironomidae assemblage structure was determined by seasonal variables such as rainfall, photoperiod and water and air temperature. These variables fluctuate annually according to the seasons, acting as exogenous signals that operate on the larva and pupa, affecting the development pace and the timing of life history in chironomids (Tokeshi 1995, Danks 2007). In the present study, we also observed (Fig. 3) how these variables influence life cycles, with Chironominae emerging mainly in spring-summer and Orthocladiinae in colder periods.

Temperature is one of the most influential environmental variables, since it plays a major role in metabolism control, growth and development, voltinism and behaviour (Armitage 1995, Lencioni et al. 2008). In the study on the relationship between Chironomidae emergence peaks for lotic environments reported by Coffman (1973), it was observed that the beginning of Orthocladiinae emergence was associated with the increase in water temperature in early spring. On the other hand, the same author reported that the Chironomini and Tanypodinae peaks were associated mainly to summer, when water temperature reached maximum annual values, whereas Tanytarsini peaks were associated to maximum fluctuations of water temperature.

Photoperiod also acts on the larval diapause and, together with temperature, can determine the time of emergence (Armitage 1995). Temperature increase and photoperiod are associated to the beginning of spring, therefore, when accumulated day-degrees exceed their specific threshold, chironomids that survive winters manage to complete their development (Mundie 1957).

Our study found negative correlations of the different subfamilies with the nutrients. Nutrients are inorganic materials necessary to life, of which P and N are the primary nutrients that limit biological activity within lotic ecosystems (Allan & Castillo 2007). However, above the threshold, they mainly affect the most sensitive species

(Merrit et al. 2008). As a consequence of their great differences in tolerance to eutrophication and organic enrichment, chironomids are markers of nutrient presence (Lindegaard 1995).

The study of invertebrate life cycles not only provides important information on the autecology of species, but also enables the analysis of the development, emergence and production response to potential impacts of climate change, a worldwide concern that has been increasing in recent years. Insects can respond to climate change by modifying their geographic range, or they can adapt through altered phenology, such as earlier or later emergence, life stages taking place at different times or by increasing the number of generations per year. These changes can lead to new interactions and changes in community structure. Climate change will affect insects due to their dependence on environmental temperature for controlling their physiological functions (Maran & Pelini 2016). Therefore, knowledge of the phenology and voltinism of Chironomidae species will assist prediction of the responses of these insects to climate change, improving our understanding of what these changes affecting ecosystems may be like.

Exuviae collection is a way of measuring taxon presence, life history, population size and productivity, since it marks adult emergence as the final point of the life cycle and successful habitat use by the immature stages (Ferrington et al. 2008). Measurement of emergence in aquatic insects has been used for taxonomic and ecological studies as well as studies on productivity and biological assessment. Better taxonomic resolution enables more complete taxon lists to be created and reduces the problems associated with drawing conclusions from results including taxa that have been grouped with potentially different tolerances and life histories (Raunio et al. 2007a). Furthermore, the CPET is efficient in phenological analysis, because it enables the collection of species with short periods of activity, which would otherwise be lost.

From the different aspects analysed in the present study, the first contributions arise from the application of the pupal exuviae technique on chironomids from Pampean streams. Species composition was accurately recorded, broadening the systematic annual list for the region and providing information on the phenological characteristics of the chironomid community from first order Pampean streams. Furthermore, we established that the environmental variables structuring Chironomidae emergence, mainly temperature, determine the phenology and voltinism of the study species in the period analysed.

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SUPPLEMENTARY MATERIAL

Tables SI, SII.

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JPZA, LM and MD conceived and designed the study. JPZA, LNSRC and AS collected the samples. JPZA compiled the database, performed the analyses, and interpreted the results. JPZA wrote the first draft with substantial inputs from all authors. All authors reviewed and commented on the manuscript and approved the final version.