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RESEARCH

Exploring the range of *Culex* mosquitoes in Western Argentinean Patagonia, unveiling the presence of *Culex pipiens* bioform *pipiens* in South America

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Abstract

Culicids in Argentinean Patagonia are characterized by low species diversity and adaptation to extreme environmental conditions, yet few studies have been conducted in the region. To further assess the occurrence of Culicidae in Western Patagonia, and in particular the presence of *Culex pipiens* bioforms at the southernmost extent of their distribution, immature and adult specimens were collected aboveground across various land uses located in shrubland, steppe, and deciduous forest between 38.96 and 46.55°S. Mosquitoes were reported at 35 of the 105 inspected sites. Five species from the genus *Culex* were identified, all of which were present in the steppe and the forest, while only *Cx. apicinus* and members of the *Cx. pipiens* complex were collected in the shrubland. Within the latter, a total of 150 specimens were molecularly identified by PCR amplification of Ace-2 and CQ11 loci. The first-to-date occurrence of bioform *pipiens* in South America is reported, along with the first records of *Cx. quinquefasciatus* signatures in Patagonia. In addition, the distribution of *Cx. acharistus* and *Cx. dolosus* as south as Santa Cruz province is expanded, and the first record of *Cx. eduardoi* in Río Negro province is provided. Immature specimens of *Cx. pipiens* were conspicuous in human-made aquatic habitats (both containers and in the ground), while *Cx. acharistus* was more prominent in artificial containers and *Cx. eduardoi* was mainly in ground habitats, either natural or human-made. These findings provide valuable insights into the distribution and ecological roles of these mosquito species in a region of extreme environmental conditions.

Keywords *Culex pipiens* complex · *Culex acharistus* · *Culex dolosus* · *Culex eduardoi* · Larval habitat type · Mosquito distribution

Introduction

Mosquitoes (Diptera: Culicidae) represent a highly diverse group of insects, showcasing a wide array of adaptations to thrive in various climates and even extreme conditions. This adaptability allows them to inhabit almost every corner of the globe with the exception of Antarctica and a few islands (Service 1993). In southern South America, Patagonia is a vast territory shared by Argentina and Chile that represents the southernmost continental extension. On the Argentinean side, this region of 787,200 km^2 and almost 2000 km long N-S includes a wide range of climatic and edaphic conditions, resulting in various vegetation formations (Cabrera and Willink 1973).

Mosquito research in Argentinean Patagonia has a long but scant history, dating back to the late 1920s when immatures of *Aedes* and *Culex* were recorded during an expedition by Edwards and Shannon (1927). After a considerable

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hiatus, Bachmann and Bejarano (1960) resumed the work, followed by Mitchell et al. (1984) and Almirón et al. (1995), the latter focusing on the *Cx. pipiens* complex along the coast. A few years later, Burroni et al. (2007) reported the presence of *Cx. eduardoi* in Chubut province. The compilation by Rossi and Vezzani (2011) listed 15 species for the region, primarily represented by the *Culex* genus and including species from three subgenera, some of which are of sanitary relevance. Subsequently, the yellow fever mosquito, *Aedes aegypti*, was reported at the northernmost border of Patagonia (Grech et al. 2012; Rubio et al. 2020), expanding the total number of mosquito species to 16 in the region (Rossi 2015), out of the 246 reported for the entire country (Stein et al. 2018). In more recent studies, Grech et al. (2019) characterized the larval ecology of seven species belonging to the genera *Culex* and *Aedes* in natural temporary pools, highlighting the prominence of *Culex apicinus*, *Cx. acharistus*, and *Aedes albifasciatus*. Furthermore, Grech et al. (2023) revisited the distribution of *Cx. acharistus* in Patagonia.

The previous historical overview provides evidence that, despite their significance in terms of public health concerns, mosquitoes inhabiting human-made aquatic habitats have not been thoroughly investigated. Of them, the members of the *Cx. pipiens* complex are ubiquitous vectors of diseases that afflict humans, companion and productive animals, and wildlife (Farajollahi et al. 2011). In particular, they are considered primary vectors of West Nile virus (WNV), the most widely distributed arbovirus in the world. After its first detection in New York City in 1999, it spread rapidly across North America, causing multiple outbreaks in human and bird populations, and accounting for over seven million human infections only in the USA in less than two decades (Hadfield et al. 2019). Another arboviral disease, caused by the St. Louis Encephalitis virus (SLEV), coexists with WNV in the Americas, with eight genotypes in a broad geographical range and causing sporadical epidemics from the USA to central Argentina (Díaz et al. 2018). In the USA, the members of the *Cx. pipiens* complex have been incriminated as the primary vectors of the urban SLEV cycle (Reisen 2003).

The nominal species of the complex, *Cx. pipiens* L., occurs in cold and temperate regions and has two distinct bioforms, named *pipiens* and *molestus*. Whereas *Cx. pipiens* f. *pipiens* diapauses, requires a blood meal to lay eggs (anautogeny), and is unable to mate in confined spaces (eurygamy), *Cx. pipiens* f. *molestus* does not diapause, is able to lay its first batch of eggs without a blood meal (autogeny), and mates in confined spaces (stenogamy). The combination of stenogamy and autogeny enables *Cx. pipiens* f. *molestus* to breed in underground habitats; therefore, this bioform was originally believed to be restricted to such environments. However, more recently, aboveground populations have been documented, even at high latitudes in both hemispheres (e.g., Smith et al. 2005; Cardo et al. 2020a). The two bioforms are genetically isolated in Northern Europe, but there is clear evidence of hybridization in North America. In Patagonia, the first records of *Cx. pipiens* complex were performed in earlies 1960s (Bachmann and Bejarano 1960), followed by additional reports in 1980s–1990s (Mitchell et al. 1984; Almirón et al. 1995). Until then, all specimens had been identified by morphological or enzymatic techniques; therefore, no bioform differentiation was available (Almirón et al. 1995; Humeres et al. 1998). Cardo et al. (2020a) were the first to molecularly biotype *Cx. pipiens* specimens from east Patagonia, recording only the *molestus* form as far south as Comodoro Rivadavia (45.87°S). Further complicating the epidemiologic landscape, *Cx. quinquefasciatus* Say, a tropical to temperate species, hybridizes extensively with *Cx. pipiens* bioforms (Fonseca et al. 2004). Although it has not been reported in Patagonia (Cardo et al. 2020a), it is known to occur in provinces bordering to the north (Rossi 2015; Cardo et al. 2020b). *Cx. quinquefasciatus* is non-diapausing, anautogenous, and stenogamous, and these ecophysiological differences hold significant implications for disease transmission.

Given the limited information on Culicidae in Western Argentinean Patagonia, especially in relation to human-made aquatic habitats, and the absence of *Cx. pipiens* f. *pipiens* in prior studies along the eastern coast, this study aimed to achieve the following objectives: (1) investigate mosquito presence in various biomes across this region; (2) unravel the occurrence of *Cx. pipiens* bioforms in the country through molecular techniques. The present work includes detailed observations on mosquitos' habitat collections and, notably, marked the first report of *Cx. pipiens* f. *pipiens* in South America, carrying substantial implications for arboviral transmission.

Methods

Study area and fieldwork

The study was performed in the western fringe of Argentinean Patagonia, in a transect from the north-center (Plottier 38.96°S, Neuquén province) to the south-west (Los Antiguos 46.55°S, Santa Cruz province) (Fig. 1). Throughout the nearly 1300 km of sampling, a transition was encompassed from shrubland vegetation in the extreme north to steppe terrain in the south-east and extreme west, and deciduous forest in between (Fig. 1B). Sampling sites in the shrubland presented mean annual temperatures in the range 12.7–14.6 °C, scarce annual precipitation (150–250 mm), and consequently negative hydric balance (Bianchi and Cravero 2010). This biome is characterized by a sparse xerophytic steppe (*Larrea* and *Parkinsonia* spp.) with several strata. The lower stratum

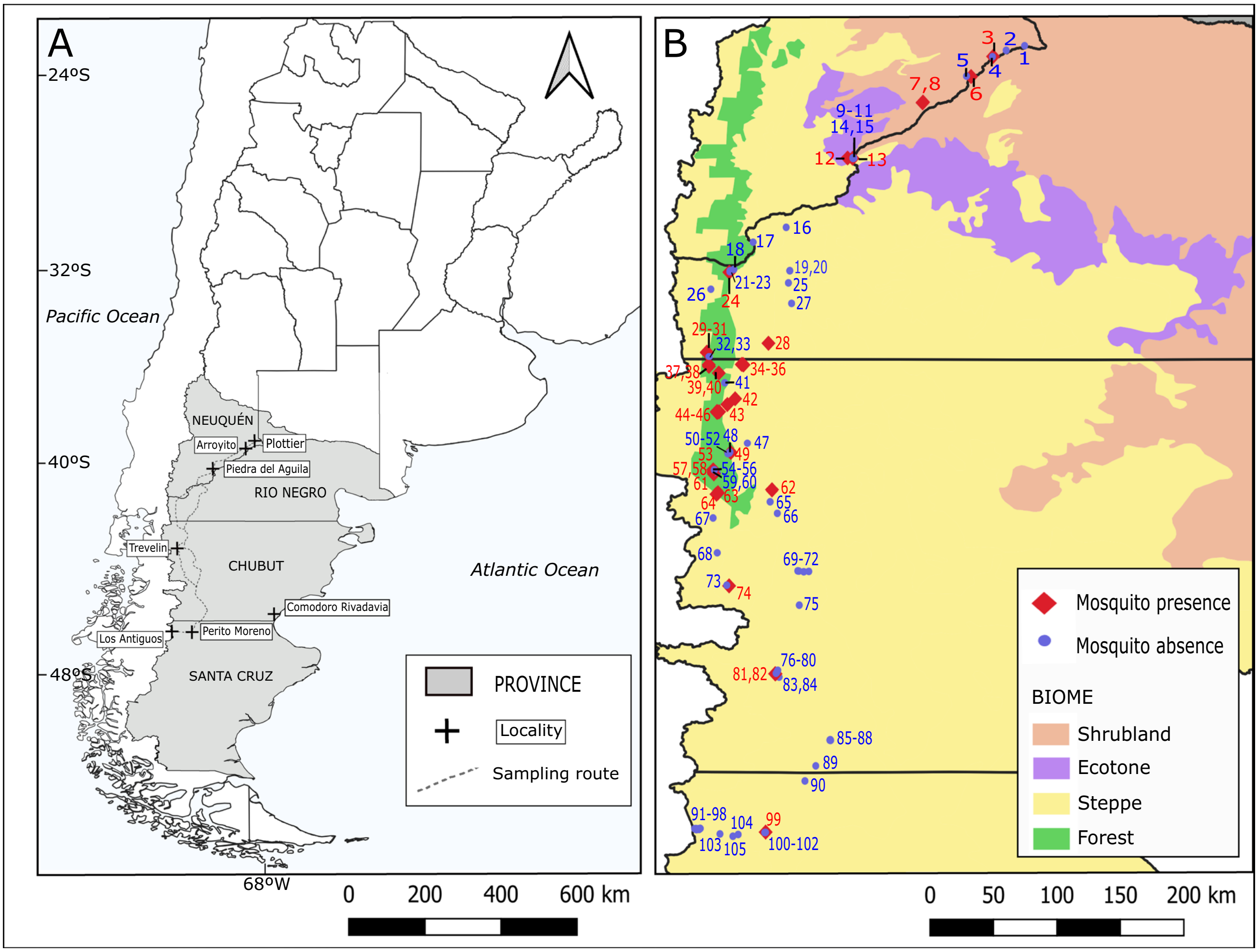


Fig. 1 Occurrence of Culicidae in Western Patagonia, Argentina. (**A**) Sampled provinces in Argentinean Patagonia; provinces names in capital letters, localities named in the text in lowercase letters. (**B**) Detail of the study area, indicating biomes; numbers next to each point indicate sampling locations; mosquito presence and absence are indicated in red diamonds and blue dots, respectively (see Table 1 for details)

(< 0.5 m) consists of grasses, herbs, and low shrubs with a coverage of 10–20%. The low and middle strata (0.5–1.5 m) seldom exceed 40% coverage, and the upper stratum (up to 2 m) is highly dispersed (Oyarzabal et al. 2018). Transitioning into the steppe, in areas with annual precipitation ranging between 200 and 300 mm, the typical vegetation is dominated by grasses from the genera *Pappostipa* and *Poa*, along with shrubs like *Adesmia volckmannii* and *Berberis microphylla*, which reach heights between 60 and 180 cm (Oyarzabal et al. 2018). In regions with annual precipitation ranging from 300 to 600 mm, the grassy steppe increases its coverage and encroaches into the eastern sector of the deciduous forest in a broad patchy ecotone. These areas have soils relatively rich in organic matter and good water retention capacity. Sampling in the steppe was performed in a broad latitudinal span, including sites in three provinces (Fig. 1B) and mean annual temperatures between 6.7 and 11.1 °C (Bianchi and Cravero 2010). The deciduous forest occupies a narrow and limited strip in the westernmost part of our country, characterized by medium-to-small-sized leaves or scale-like foliage, primarily *Notophagus* spp. (Oyarzabal et al. 2018). In sampled sites, the mean temperature ranged from 7.8 to 10.8 °C, and cumulative annual precipitation varied from 500 to 950 mm (Bianchi and Cravero 2010).

The demographic profile of Argentinean Patagonia reveals the lowest population density in the country (3.3 inh/km^2, INDEC - Instituto Nacional de Estadística y Censos 2023), with extensive sparsely inhabited areas. Meanwhile, urban centers, agricultural areas, tourist destinations, and extractive zones act as population focal points. The primary use in the semiarid shrubland ecosystems is livestock grazing, with a focus on sheep and cows for wool and meat production (Zeberio et al. 2018). The steppe is known for sheep farming, while some areas also engage in fruit and vegetable

Table 1 Culicidae by sampling site in Western Patagonia, Argentina (site numbers correspond to those in Fig. 1B)

Site *n*°	Province	Locality	Biome	Land use	Immature collection		Adult collection	*Cx. pipiens* complex				*Cx. acharistus*	*Cx. apicinus*	*Cx. dolosus*	*Cx. eduardoi*
					Stage	Habitat		p	m	h	q				
3	Neuquén	Arroyito	Shrubland	Fuel station	LP	AC – 200 L metal can	-		✓						
6		El Chocón	Shrubland	Recreational	LP	HG – 3 m wide stone pool	-		✓†	✓	✓				
7		Picún Leufú	Shrubland	Tire-repair shop	LP	AC – 200 L bathtub	Bathtub edges	✓	✓	✓	✓		✓		
8			Shrubland	Tire-repair shop	-	-	Backyard	✓			✓				
12		Piedra del Águila	Shrubland	Dwelling	-	-	Resting on inner walls						✓		
13			Shrubland*	Recreational	-	-	Beneath grill	✓	✓	✓					
24	Río Negro	Bariloche	Forest	Cemetery	-	-	Tool storage room	✓	✓	✓					
28		Ñorquin-có	Steppe	Tire-repair shop	-	-	Inner side of the plastic Lone	✓	✓	✓					
29		El Bolsón	Forest	Road margin	L	HG – artificial ditch	Inside ditch			✓					✓
30			Forest	Cemetery	LP	HG – water-logged graves	Broken water tank	✓†	✓†	✓			✓		
31			Forest	Road margin	L	HG – artificial ditch	Inside ditch								✓
34	Chubut	Maitén	Steppe	Cemetery	L	HG – sink	Entrance hallway	✓	✓						
35			Steppe	Tire-repair shop	-	-	Inner walls	✓							
36			Steppe	Dwelling	LP	AC – 20 L cut plastic pot	Bathroom	✓†	✓†	✓		✓			
37		El Hoyo	Forest	Road margin	LP	HG – artificial ditch	-								✓
38			Forest	Tire-repair shop	L	AC – 200 L bathtub	-	✓	✓	✓		✓			
39			Forest	Tire-repair shop	LP	AC – discarded tire	-	✓		✓		✓		✓	
40		Epuyén	Forest	Cemetery	LP	AC – flower vases	-	✓	✓	✓		✓	✓		
42			Steppe	River margin	LP	NG – overflow temporary pool	-					✓			✓
43		Cholila	Steppe	River margin	LP	NG – overflow temporary pool	-					✓			✓
44			Forest	River margin	LP	NG – overflow temporary pool	-					✓			✓
45			Forest	Tire-repair shop	L	AC – 200 L bathtub	-					✓			
46			Forest	Tire-repair shop	L	AC – 200 L bathtub	-					✓			
49		Esquel	Forest	Cemetery	LP	AC – flower vases	Entrance hallway, bathroom	✓				✓	✓	✓	
53		Trevelin	Forest	Recreational	L	NG – flooded ground	-								✓
57			Forest	Dwelling	LP	AC – cement fountain	-	✓	✓†	✓					✓
58			Forest	Road margin	LP	HG – artificial ditch	-	✓	✓	✓		✓			✓
61			Forest	Road margin	L	HG – soil ditch	Inside ditch	✓†	✓						✓
62		RN 40	Steppe	Road margin	L	NG – flooded ground	-							✓	✓
63		RP 17	Forest	Road margin	L	NG – temporary pool	-								✓
64			Forest	Road margin	L	NG – flooded ground	-								✓
74		Río Pico	Steppe	Road margin	LP	NG – flooded ground	-								✓

Table 1 (continued)

Site *n*°	Province	Locality	Biome	Land use	Immature collection		Adult collection	*Cx. pipiens* complex				*Cx. acharistus*	*Cx. apicinus*	*Cx. dolosus*	*Cx. eduardoi*
					Stage	Habitat		p	m	h	q				
81		Alto Río Senguer	Steppe	River margin	L	NG – flooded ground	-								✓
82			Steppe	River margin	LP	NG – flooded ground	-					✓			✓
99	Santa Cruz	Perito Moreno	Steppe	Cemetery	LP	AC – paint bucket	-					✓	✓	✓	

Biome categories follow Oyarzabal et al. (2018). Within the immature collection, stages are L for larvae and P for pupae; habitats are AC for artificial containers, HG for human-made ground habitats, and NG for natural ground habitats. Within the *Cx. pipiens* complex, the occurrence of *Cx. pipiens pipiens* (p), *Cx. pipiens molestus* (m), hybrids between the two bioforms (h), and hybrids including *Cx. quinquefasciatus* signatures (q) is informed in separate columns

*Ecotone shrubland-steepe

†1 specimen sequenced, ID confirmed

cultivation. The forested areas are important for the forestry industry, including timber and non-timber forest products. Additionally, activities like livestock farming, fishing, and tourism are developed. In the western arid lands, goat farming is significant. Some areas also have a history of mineral mining, and oil and gas extraction are growing activities.

Mosquito specimens were collected during February 2023 in recreational areas (campings, clubs, parks), fuel stations, cemeteries, tire-repair shops, dwellings, river margins, and road margins. Each site was geo-referenced, and an active search for mosquito specimens was performed. Adults were caught with hand nets, manual and battery-powered hand aspirators (if required, an extensor pole was attached), both outdoors and indoors. Immatures were collected using various techniques according to the type of aquatic habitat. In artificial containers such as discarded vehicle tires, uncovered water tanks, and flower vases, dipping was performed with a 200-ml ladle and a fine mesh strainer. In ground habitats, either natural (e.g., temporary pools) or human-made (e.g., ditches), sweeping was performed using fine mesh nets (10 × 7 cm) and complemented with dipping with a white plastic tray (30 × 15 × 5 cm). Larvae and pupae were separated in the field; larvae were fixed in ethanol 96%, while pupae were transported alive during the collection day and aspirated if they emerged before night; otherwise, they were discarded by fixing in ethanol 96%. Adults collected in the field and those that emerged from pupae were preserved at – 16 °C in a portable car freezer until processing, which occurred during the night of each collection day. Both adults and third-fourth instar larvae were morphologically identified under an 80 × stereoscopic microscope using dichotomic keys (Darsie 1985) and species descriptions (e.g., Laurito et al. 2009). Adult specimens assigned to the *Cx. pipiens* complex were further fixed in ethanol 96% for preservation until molecular analyses.

Laboratory work

Individual larvae and adults of the *Cx. pipiens* complex were ground with sterilized mortar and pestle, and genomic DNA was extracted using the EasyPure Genomic DNA extraction kit (Transgen Biotech). Specimen identification followed established PCR protocols for the amplification of the second intron of the Ace-2 nuclear gene (Smith and Fonseca 2004) and the 5′ flanking region of microsatellite locus CQ11 (Bahnck and Fonseca 2006). The first protocol amplifies a 610-bp band for *Cx. pipiens* and a 274-bp band for *Cx. quinquefasciatus*, whereas the simultaneous presence of both bands is indicative of hybrid signatures. The second protocol distinguishes between both forms of *Cx. pipiens* amplifying a 250-bp band for *molestus* and a 190–200-bp band for *pipiens*; again, the presence of both bands

Table 2 Number (proportion) of positive sites per province, biome, and land use for each mosquito species/bioform/hybrid collected in Western Patagonia, Argentina

	Cx. p. pipiens (16)	*Cx. p. molestus* (14)	Bioforms hybrids (13)	*Cx. quinq*-hybrids (3)	*Cx. acharistus* (13)	*Cx. apicinus* (6)	*Cx. dolosus* (4)	*Cx. eduardoi* (16)
Province								
Neuquén (15)	3 (0.20)^{a}	4 (0.27)^{a}	3 (0.20)^{a}	3 (0.20)		2 (0.13)		
Río Negro (18)	3 (0.17)^{a}	3 (0.17)^{a}	4 (0.22)^{a}			1 (0.06)		2 (0.11)^{a}
Chubut (56)	10 (0.18)^{a}	7 (0.13)^{a}	6 (0.11)^{a}		12 (0.21)^{a}	2 (0.04)	3 (0.05)	14 (0.25)^{a}
Santa Cruz (16)					1 (0.06)^{a}	1 (0.06)	1 (0.06)	
Biome								
Shrubland (15*)	3 (0.20)^{a}	4 (0.27)^{a}	3 (0.20)^{a}	3 (0.20)		2 (0.13)		
Steppe (56)	4 (0.07)^{b}	3 (0.05)^{b}	2 (0.04)^{b}		5 (0.09)^{a}	1 (0.02)	2 (0.04)	6 (0.11)^{a}
Forest (34)	9 (0.26)^{a}	7 (0.21)^{a}	8 (0.24)^{a}		8 (0.24)^{a}	3 (0.09)	2 (0.06)	10 (0.29)^{b}
Land use								
Road margin (29)	2 (0.07)	2 (0.07)	2 (0.07)		1 (0.03)		1 (0.03)	9 (0.31)
Fuel station (21)		1 (0.05)						
Tire-repair shop (21)	6 (0.29)	3 (0.14)	4 (0.19)	2 (0.10)	4 (0.19)	1 (0.05)	1 (0.05)	
Cemetery (11)	5 (0.45)	4 (0.36)	3 (0.27)		3 (0.27)	4 (0.36)	2 (0.18)	
Recreational area (8)	1 (0.13)	2 (0.25)	2 (0.25)	1 (0.13)				1 (0.13)
River margin (8)					4 (0.50)			5 (0.63)
Dwelling (7)	2 (0.29)	2 (0.29)	2 (0.29)		1 (0.14)	1 (0.14)		1 (0.14)

In the first column, the number of inspected sites is informed between brackets next to each category, while for species/bioforms/hybrids, the total number of positive sites is informed between brackets. The shrubland biome includes 5 sites in the ecotone shrubland steppe (marked with an asterisk; see text for details). Different superscript letters within each column and factor (biome or province) indicate significant differences in univariate GLM, only evaluated for mosquito species/bioforms/hybrids present in ≥ 10 sites

indicates hybrid signatures. A negative (distilled water) control and positive controls from *Cx. pipiens* f. *pipiens* (Southern France), *Cx. pipiens* f. *molestus* (England), and *Cx. quinquefasciatus* (Indonesia) were included in all runs. A 5-µl aliquot of each amplified product mixed with 1 µl of loading buffer 6 × was electrophoresed in a 2% agarose gel containing ethidium bromide (0.5 µg/mL) and 0.5X TBE buffer. Bands were visualized under a gel UV transilluminator. DNA ladders of 50 bp and 100 bp precision were run in parallel to allow size estimation of observed bands.

Amplified PCR products of seven specimens were purified and sequenced in an ABI 3130xl Genetic Analyzer (Applied Biosystems) by a third-party provider. Sequences were edited using ApE v2.0.55 and compared to known sequences by a BLAST search comparison with the GenBank DNA database (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Statistical analysis

The biome associated with each geo-referenced site was extracted from the publicly accessible shapefile provided by Oyarzabal et al. (2018). The sites situated within the ecotone between shrubland and steppe (sites 9–11, 13, and 14 in Fig. 1B) were categorized as part of the shrubland. This decision was made because, within the same locality (Piedra del Águila, Neuquén province), there were sampling sites representing both pure shrubland (sites 12 and 15) and the ecotone, and all sites were equivalent and intermixed.

Climatic variables considered were mean annual temperature (T_mean), mean temperature of the coldest trimester (T_cold), mean cumulative precipitation (P_annu), and hydric balance (HB). Values were extracted for each site from the corresponding raster layers (0.001° resolution) openly accessible at Bianchi and Cravero (2010).

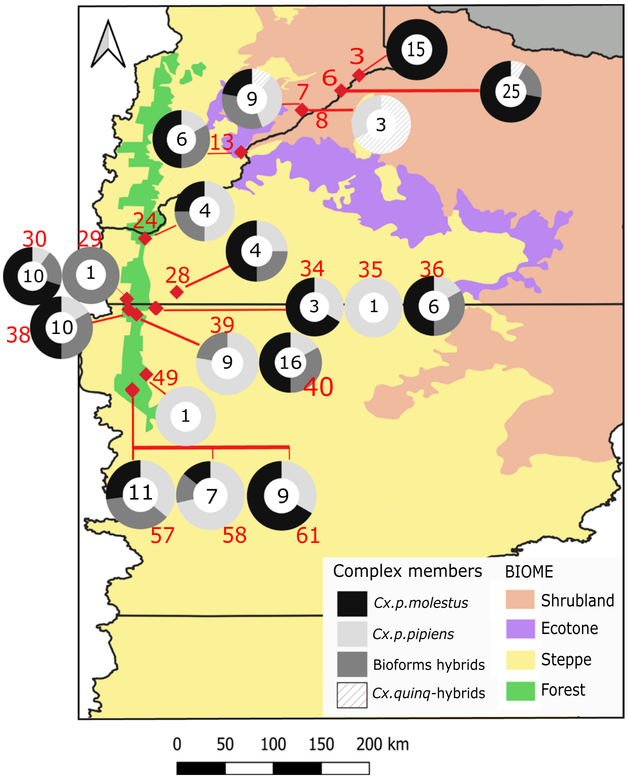


Fig. 2 Occurrence of the members of the *Culex pipiens* complex in Western Patagonia, Argentina. The relative composition of *Cx. pipiens molestus* (*Cx. p. molestus*), *Cx. pipiens pipiens* (*Cx. p. pipiens*), hybrids between the two bioforms (Bioforms hybrids), and hybrids including *Cx. quinquefasciatus* signatures (*Cx. quinq*-hybrids) is informed as a pie chart for each site. The number of identified specimens at each site is reported inside the chart. Red numbers next to each diamond indicate collection sites (see Table 1 for details)

Given the high correlation observed between the pairs T_mean – T_cold and P_annu – HB ($r^2 > 0.95$), the first variable from each pair was retained for analysis. This choice was made for the sake of simplicity in data acquisition and interpretation.

Generalized linear models (GLM) were run to assess the relationship between mosquito presence and environmental factors. All models were considered at the site scale, i.e., the site was the minimum analysis unit. Explanatory variables were T_mean, P_annu, biome (3 levels; shrubland, steppe, and forest), and province (4 levels; Neuquén, Río Negro, Chubut, and Santa Cruz). Land use could not be evaluated due to the high number of categories (7) and zeros in several categories for the occurrence of a given species. A stepwise forward procedure was conducted, in which explanatory variables were entered one by one along with all two-way interactions. A term was retained in the model if it reduced the value of the Akaike information criterion (AIC) by more than 2 units (Zuur et al. 2009).

Occurrence models, i.e., presence/absence, were run for each species/bioform/hybrid present in ≥ 10 sampled sites using binomial error distribution (link log). Then, the identity and relative proportion of both bioforms of *Cx. pipiens* at each positive site were summarized in a Bioform Index (BI), calculated as.

$$\mathrm{BI} = \frac{\text{N° of } \textit{Cx. pipiens molestus} \text{ specimens} - \text{N° of } \textit{Cx. pipiens pipiens} \text{ specimens}}{\text{N° of } \textit{Cx. pipiens molestus} \text{ specimens} + \text{N° of } \textit{Cx. pipiens pipiens} \text{ specimens}}$$

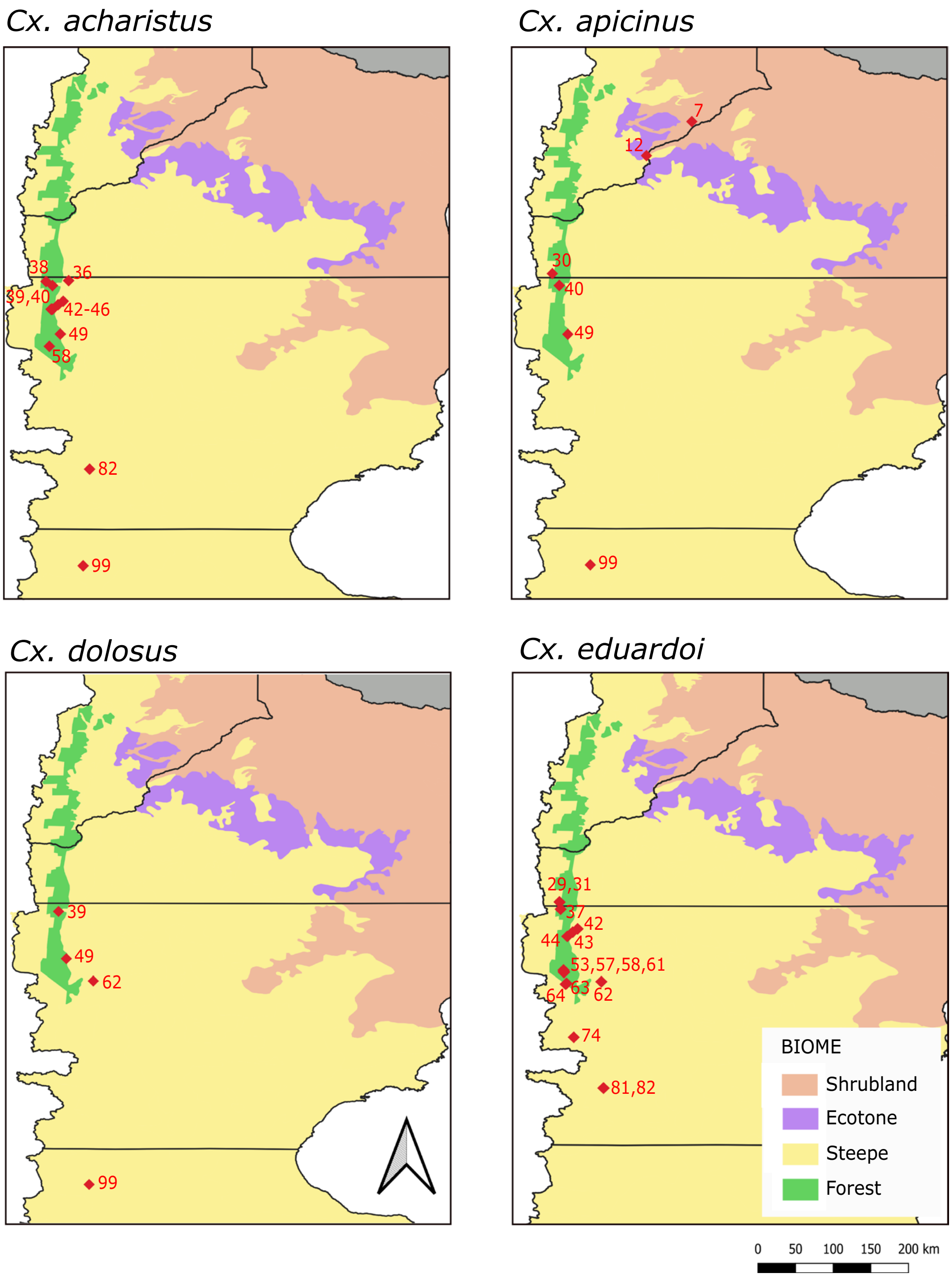
The BI took values from + 1 for pure *Cx. pipiens molestus* populations to – 1 for pure *Cx. pipiens pipiens* populations. The total number of identified specimens per site was included in the model as a weight and error distribution was Gaussian (link identity).

To search for possible segregation patterns among aquatic habitat types, a univariate GLM considering only the type of aquatic habitat as an explanatory variable (three levels: artificial container, human-made ground habitat, and natural ground habitat) was run for the occurrence of immatures of any mosquito species, as well as for each one represented in ≥ 10 total positive sites, using binomial error distribution (link log).

Finally, the association between *Cx. pipiens* bioforms in each biome was evaluated using Hurlbert's C8 association coefficient (Hurlbert 1978). Positive values indicate coexistence and can reflect a common habitat preference or attraction between both bioforms, while negative values can result from differences in habitat preference or interspecific interaction.

Results

The survey was conducted in 105 sites, resulting in the collection of adult and/or immature specimens of *Culex* mosquitoes at 35 of these sites (Table 1, Fig. 1, Supplementary Table S1). Collected species were *Cx. acharistus*, *Cx. apicinus*, *Cx. dolosus*, *Cx. eduardoi*, and both bioforms of *Cx. pipiens* and their hybrids, plus *Cx. quinquefasciatus* hybridizing with *Cx. pipiens*. Species richness was highest in the steppe and the forest, whereas only *Cx. pipiens* complex and *Cx. apicinus* were collected in the shrublands (Table 2). Although over half of the inspected sites were located in the steppe, more mosquito collections were performed in the forest (Table 2). Members of the *Cx. pipiens* complex were collected from Arroyito (site 3, 39.26°S, Neuquén province) to Trevelin (sites 57, 58, and 61, 43.11°S, Chubut province) in all land uses except river margins (Table 2, Fig. 2). Both bioforms of *Cx. pipiens* and the hybrid between the two were equally present in three of the four sampled provinces and in all biomes, with a higher presence in shrubland and forest than in the steppe (Table 2). *Cx. acharistus* was ubiquitous



Cx. acharistus

Cx. apicinus

Cx. dolosus

Cx. eduardoi

Fig. 3 Occurrence of four *Culex* species in Western Patagonia, Argentina. Red numbers next to each diamond indicate collection sites (see Table 1 for details)

in Chubut province, both in steppe and forest (Table 2), and was registered in Santa Cruz province (Perito Moreno, site 99 in Fig. 3) for the first time. *Cx. apicinus* occurred less but widely distributed, all along the extension of the study region (Fig. 3). Four collections of *Cx. dolosus* specimens were performed in Chubut and Santa Cruz provinces, marking a new provincial record for the latter (Fig. 3), while *Cx. eduardoi* was notably prevalent in Río Negro (also representing a new provincial record) and Chubut with a greater presence in the forest compared to the steppe (Fig. 3, Table 2).

Out of the 105 sites surveyed, aquatic habitats were examined at 70 of them, with 9 cases involving more than one type of habitat. A total of 425 water collections were inspected; the majority were artificial containers, followed by natural ground habitats and, lastly, human-made ground habitats (Supplementary Table S1). In general, the probability of occurrence of mosquito immatures was higher in human-made aquatic habitats (either containers or in the ground) than in natural ground habitats (Table 3). In line with this result, specimens of the *Cx. pipiens* complex were collected exclusively in human-made aquatic habitats, whereas *Cx. eduardoi* was most prevalent in both types of ground habitats, and *Cx. acharistus* was collected across all types of habitats with higher occurrence in artificial containers (Table 3).

Table 3 Number (proportion) of sites positive for immatures of each mosquito species/bioform/hybrid by type of aquatic habitat in Western Patagonia, Argentina

Type of aquatic habitat	Any mosquito species (29)	*Cx. p. pipiens* (11)	*Cx. p. molestus* (11)	Bioforms hybrids (10)	*Cx. quinq*-hybrids (2)	*Cx. acharistus* (13)	*Cx. apicinus* (5)	*Cx. dolosus* (4)	*Cx. eduardoi* (16)
Artificial container (25)	12 (0.48)^{a}	8 (0.32)^{a}	7 (0.28)^{a}	7 (0.28)^{a}	1 (0.04)	8 (0.32)^{a}	5 (0.2)	3 (0.12)	1 (0.04)^{a}
Human-made ground habitat (13)	7 (0.54)^{a}	3 (0.23)^{a}	4 (0.31)^{a}	3 (0.23)^{a}	1 (0.08)	1 (0.08)^{b}			5 (0.38)^{b}
Natural ground habitat (41)	10 (0.24)^{b}					4 (0.10)^{b}		1 (0.02)	10 (0.24)^{b}

In margins, numbers between brackets in the first column inform the total number of sites inspected that presented that category of aquatic habitats, while in the first row, they indicate the total number of sites positive for immatures per species (or bioforms or hybrids). Different superscript letters at each column indicate significant differences in univariate GLM, only evaluated for mosquito species/bioforms/hybrids present in ≥ 10 sites

A total of 150 specimens (120 larvae, 18 males, 12 females) of the *Cx. pipiens* complex were molecularly identified. Of them, *Cx. pipiens* was present all along the study area, with bioform *molestus* accounting for half of the specimens, while the remaining half was divided among pure *Cx. pipiens* f. *pipiens* and hybrids between the two forms. Four hybrid specimens of either bioform with *Cx. quinquefasciatus* were encountered at the northern extreme of the study area (sites 6–8), all located within Neuquén province (Fig. 2). The identity of three specimens of *Cx. pipiens* f. *pipiens* (sites 30, 36, and 61) and four specimens of *Cx. pipiens* f. *molestus* (sites 6, 30, 36, and 57) was corroborated by blast matches of the sequence of the CQ11 amplified PCR products; > 99% identity with GenBank reference specimens KY744217.1, DQ470148.1 and/or DQ470146.1 for *Cx. pipiens* f. *pipiens*, and with KY744220.1 and/or KY744221.1 for *Cx. pipiens* f. *molestus*.

The presence/absence of binomial GLMs for each species/bioform/hybrid ($N = 105$) indicated that the occurrence of both bioforms of *Cx. pipiens* and the hybrid between the two was positively associated with T_mean and P_annu. For their part, *Cx. acharistus* and *Cx. eduardoi* were only positively associated with P_annu (Table 4). When considering the relative proportion of bioforms *pipiens* and *molestus* at each site positive for *Cx. pipiens* ($N = 18$, as site 29 had only 1 hybrid specimen identified and was therefore excluded), the BI was higher in Neuquén than in Río Negro and Chubut, higher in shrubland than in steppe and forest,

Table 4 Generalized linear models for the occurrence of each mosquito species/bioform/hybrid collected and for the relative proportion of *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus*, calculated as the Bioform Index (see "Methods" section), in Western Patagonia, Argentina

	Cx. p. pipiens	*Cx. p. molestus*	Bioforms hybrids	*Cx. acharistus*	*Cx. eduardoi*	Bioform Index
AIC null model	91.6	84.5	80.6	80.6	91.6	43.8
Univariate models						
Province	91.8	83.8	80.2	73.7	83.5	40.8
Biome	89.1	81.4	75.4	76.8	85.3	41.7
Mean annual temperature (T_mean)	91.1	81.1 (+)	79.2	82.0	90.8	40.5 (+)
Mean cumulative precipitation (P_annu)	89.1 (+)	84.2	76.7 (+)	75.1 (+)	82.0 (+)	40.6 (−)
Multivariate model						
Terms	T_mean(+) + P_annu(+)	T_mean(+) + P_annu(+)	T_mean(+) + P_annu(+)	–	–	–
AIC	84.8	76.4	69.1	–	–	–

For univariate models, the Akaike Information Criterion (AIC) is reported along with the sign of the association for continuous variables. Differences in factor levels for categorical variables tested (province and biome) are reported in Table 2. For *Cx. acharistus*, *Cx. eduardoi*, and the Bioform Index, no significant multivariate model was obtained

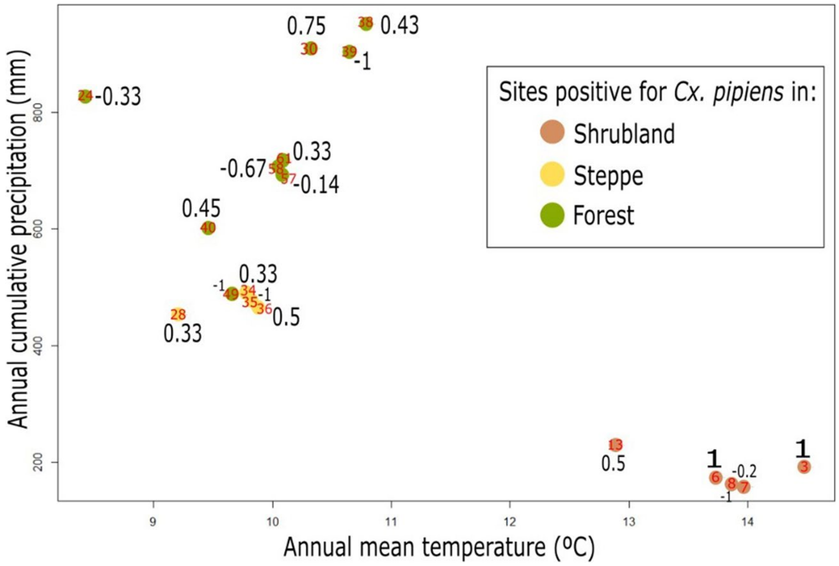


Fig. 4 Bioform Index (black numbers) for each sampling site positive for *Culex pipiens* in shrubland, steppe, and forest, as a function of mean annual temperature and cumulative annual precipitation. The size of the numbers is proportional to the number of specimens identified per site. Red numbers inside each dot indicate collection sites (see Table 1 for details)

positively associated with T_mean and negatively associated with P_annu (Fig. 4). These results are consistent in suggesting a higher proportion of bioform *molestus* in the northern extreme of the sampling region. The C8 values were 0 within the shrubland, 1 within the steppe, and 0.73 within the forest. These values reflect varying degrees of association between both bioforms based on the considered biome. Noteworthy, all these values fell within the range of zero to positive, signifying a spectrum from randomness to coexistence.

Discussion

Culicids, known for high species diversity and adaptation to extreme conditions, have seen limited study in Argentinean Patagonia. The present study provides insights into the distribution and some ecological issues of five *Culex* species collected in diverse Patagonian habitats across three biomes. As highlights, we report the first South American occurrence of bioform *pipiens*, extensively hybridizing with bioform *molestus*, while hybrid signatures including *Cx. quinquefasciatus* were recorded for the first time in Patagonia. We also provide three new species provincial records, extending the distributions of *Cx. acharistus* and *Cx. dolosus* to Santa Cruz province, and first-to-date documenting specimens of *Cx. eduardoi* in Río Negro province (see the catalog by Rossi 2015).

Enhanced by their poikilothermic nature, mosquito populations respond to variations in environmental conditions. The successful establishment of different species is influenced by the complexity of the environment, which is shaped by the combination of climatic elements, the presence of suitable aquatic habitats for immature development, and the availability of food for adults, all modulated by human modifications of the environment such as land use change and water management practices. Herein, we described variations in the first two components of heterogeneity. As expected, each species (or bioform or hybrid) was more frequently found in areas with higher temperatures and/or increased precipitation. In line with these results, the significance of temperature for *Cx. acharistus* has been recently addressed under laboratory conditions (Grech et al. 2023). When considering only positive sites for *Cx. pipiens*, we observed a trend of a higher proportion of bioform *molestus* in areas with elevated temperatures and reduced precipitation, conditions characteristic of the shrubland, while bioform *pipiens* was proportionally more abundant in areas with lower temperature and higher precipitation in the steppe and forest.

Regarding aquatic habitats, a noteworthy observation was the occupancy partitioning between *Cx. pipiens* and *Cx. eduardoi*, with the former being more prevalent in human-made habitats and the latter in natural ground habitats. Meanwhile, *Cx. acharistus* was found in various habitat types. Despite the extensive inspection of natural ground aquatic habitats across the study region, no single immature specimen of *Cx. pipiens* was encountered in this habitat type. All immature mosquitoes were found exclusively in human-made aquatic habitats, including containers and ground habitats such as ditches. This observation aligns with the findings of Grech et al. (2019) in natural temporary ponds in western Patagonia and emphasizes the role of human-mediated water management in creating a suitable environment for the members of the *Cx. pipiens* complex. Essentially, this snapshot taken in February (a dry month in the region) illustrates how anthropogenic interventions enable the colonization of a species with significant public health implications. Nevertheless, it is important to consider the possibility of *Cx. pipiens* immatures occurring in natural ground pools during the wet season, a subject that warrants attention in future research.

Complexes of sibling species present unique challenges because of the often large differences in vectorial capacity between taxa that are morphologically indistinguishable (Bahnck and Fonseca 2006). With the advent of molecular techniques in the past two decades, DNA-based rapid assays have emerged as tools to overcome the challenges of sibling species identification. The results reported herein represent first-to-date identifications of *Cx. pipiens* f. *pipiens* in its world's southernmost distribution, in a relatively high number of individuals, and along with extensive hybridization with *Cx. pipiens* f. *molestus*. Previous work in Argentina only reported the *molestus* bioform; studies in Buenos Aires province identified specimens from La Plata City (34.87°S, 57.90°W) by a full microsatellite analysis (Micieli et al. 2013), whereas the individuals collected in 13 cemeteries throughout the province were also identified as *molestus* using the CQ11 locus (Cardo et al. 2020b). In east Patagonia, both larval and adult specimens from 11 locations were also identified as bioform *molestus* (Cardo et al. 2020a). Hereby, we also reported hybrids with *Cx. quinquefasciatus* signature but could not further describe such specimens, as the composite PCR assay (Ace-2 + CQ11) fails to separate between a hybrid *Cx. quinquefasciatus*/*Cx. pipiens* f. *pipiens* from a specimen that also has *Cx. pipiens* f. *molestus* ancestry (Bahnck and Fonseca 2006). This is because the primer for bioform *molestus* in the CQ11 protocol also binds in specimens of *Cx. quinquefasciatus*, resulting in a band of equivalent size. Also, although the CQ11 rapid assay accurately identifies all first-generation hybrids and is therefore considered a reliable diagnostic method (Di Luca et al. 2016), backcrossing events result in recombination between loci. This leads to the independent assortment of the markers within hybrids, and as a result, hybrid specimens may lose diagnostic bands (Bahnck and Fonseca 2006). For these reasons, interpretations regarding the identity of the members of the *Cx. pipiens* complex should be made at the population rather than the individual level, acknowledging that the genetic structure of *Cx. pipiens* is far more complex and cannot be fully depicted by a uni-locus analysis (Bahnck and Fonseca 2006; McAbee et al. 2008).

Among the mosquitoes collected in Patagonia, *Cx. pipiens* is a worldwide recognized vector of WNV and SLEV and of the dog heartworm *Dirofilaria immitis* (Díaz et al. 2008, 2018; Vezzani et al. 2011), while *Cx. apicinus* has been found naturally infected with SLEV (Díaz et al. 2012). Both arboviruses circulate within an enzootic cycle among birds in which mammals are dead-end hosts that may develop disease symptoms. In Argentina, few WNV human cases have been reported, but the virus has been isolated from sick horses (Morales et al. 2006) and antibodies detected in resident wild birds (Díaz et al. 2008, 2016). As for SLEV, human cases were sporadic until an unprecedented outbreak occurred in 2005, followed by case clusters in subsequent years (Díaz et al. 2018; BIV 2019). Although no human cases of either disease have been reported in Patagonia, these arboviruses are prevalent throughout North America, even at latitudes similar to, or higher than, those in the southern counterpart (Díaz et al. 2018). Moreover, recent blood-feeding studies in Buenos Aires province have reported that both *Cx. quinquefasciatus* and *Cx. pipiens* f. *molestus* exhibit opportunistic behavior, readily feeding on birds, horses, and humans when given the opportunity (Cardo et al. 2023). Furthermore, during the early 1980s, an epizootic outbreak of Western equine encephalitis involving multiple human cases was documented in Northern Patagonia (Mitchell et al. 1985). While *Aedes albifasciatus* was then incriminated as the vector, this historic fact underscores that the environmental conditions conducive to arbovirus transmission are met, at least in the northern part of Patagonia.

The occurrence of mosquitoes throughout Western Argentinean Patagonia highlights their ecological resilience and their critical role in various ecosystems. Further studies on the ecology of this small group of mosquitoes will not only advance our comprehension of mosquito behavior in challenging environmental settings but also offer valuable insights for evaluating the transmission risk evolution of mosquito-borne pathogens in the Patagonian region.

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Data availability The full collection of data performed in this study is provided in Supplementary information.

Declarations

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Declaration of generative AI and AI-assisted technologies in the writing process During the preparation of this work, the authors used GPT 3.5/OpenAI in order to revise language grammar and refine writing style. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

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