Advances in the knowledge of *Echinoparyphium megacirrus* and *Echinostoma* sp. (Digenea: Echinostomatidae) parasites of *Diplodon chilensis* (Pelecypoda) in Patagonia (Argentina)

Avances en el conocimiento de *Echinoparyphium megacirrus* y *Echinostoma* sp. (Digenea: Echinostomatidae) parásitos de *Diplodon chilensis* (Pelecypoda) en Patagonia (Argentina)

Flores, Verónica[1] and Semenas, Liliana[1]

ABSTRACT: *Diplodon chilensis* (Pelecypoda, Hyriidae) is the only species present in the Patagonian Region of the Neotropical endemic genus *Diplodon*. Metacercariae of genera *Echinostoma* and *Echinoparyphium* have been found in this bivalve species and in the snail *Lymnaea viatrix*. The aim of this work was to evaluate the characteristics of the infestations and the geographic distribution of *Echinoparyphium megacirrus* and *Echinostoma* sp., parasites of *D. chilensis* in Andean-Patagonian environments and to advance in the knowledge *Echinostoma* sp. A total of 19 environments (39°06'S - 42°36'S) were sampled in order to collect specimens of *D. chilensis* to record the presence of metacercariae and to perform experimental infestations in *Gallus gallus domesticus* with parasitized viscera. The distribution range of *E. megacirrus* and *Echinostoma* sp. was determined by the study of metacercariae in natural environments, and by experimental ovigerous adults obtained in infestations with *G.g. domesticus*. Both species of Echinostomatidae were located mainly in the pericardial cavity, and in hepatopancreas and, gonads of the moluscan host. The measurements and morphology of the metacercariae and adults of *E. megacirrus* coincide with those of the original description. For *Echinostoma* sp. metacercariae, diameter and thickness of cyst wall, and size and distribution of the crown spines are different from those previously described in *D. chilensis.* It is the first time that ovigerous specimens of *Echinostoma* from experimental infestations were obtained, indicating that *D. chilensis* is a suitable secondary host for this digenean species. Comparisons of morphology and measurements of the experimental adults obtained with other species of *Echinostoma* with 37 spines collar, would assign them to *Echinostoma chloephagae* previously described in the native *Chloephaga picta leucoptera*, although the spines in our specimens are smaller. Comparing the presence and infestation values of both metacercariae, *E. megacirrus* has a wider distribution range, is recorded in more environments and presents higher prevalences.

Keywords: Digenea, Hyriidae, metacercariae, experimental infestations, South of Argentina.

RESUMEN: *Diplodon chilensis* (Hyriidae, Pelecypoda) es la única especie del género endémico neotropical *Diplodon* presente en la Región Patagónica. Metacercarias de los géneros *Echinostoma* y *Echinoparyphium* se encontraron en este bivalvo y en el caracol *Lymnaea viatrix*. El objetivo de este trabajo fue evaluar las características de las infecciones y la distribución geográfica de los digeneos *Echinoparyphium megacirrus* y *Echinostoma* sp. parásitos de *D. chilensis* en ambientes andino-patagónicos y avanzar en el conocimiento de *Echinostoma* sp. Se muestrearon especímenes de *D. chilensis* en 19 ambientes (39°06'S - 42°36´S) para registrar el rango de distribución de las metacercarias y para realizar infecciones experimentales en *Gallus gallus domesticus* con vísceras parasitadas. La presencia de *E. megacirrus* y de *Echinostoma* sp. se determinó por el estudio de las metacercarias en ambientes naturales, localizándose ambas principalmente en cavidad pericárdica y además, en hepatopáncreas y gónadas. Se obtuvieron adultos ovígeros experimentales de *E. megacirrus* y de *Echinostoma* sp., siendo esta la primera vez que se obtienen especímenes ovígeros de *Echinostoma* sp., a partir de *D. chilensis*, lo que indicaría que este bivalvo es un hospedador secundario adecuado para esta especie. Las medidas y la morfología de las metacercarias y de los adultos ovígeros experimentales de *E. megacirrus* coinciden con las de la descripción original. Las metacercarias de *Echinostoma* sp. se diferencian de las previamente descriptas en *D. chilensis* por el diámetro y el grosor de la pared del quiste

[1] Laboratorio de Parasitología. Instituto de Biodiversidad y Medio Ambiente (INIBIOMA-Universidad Nacional del Comahue-CONICET). Avda. Quintral 1250. 8400, Bariloche, Argentina.

Correspondencia: veronicaroxanaflores@gmail.com

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y por el tamaño y distribución de las espinas de la corona. La comparación morfológica y morfométrica de los adultos experimentales obtenidos con otras especies de *Echinostoma* con 37 espinas en el collar, permitirían asignarlos a *Echinostoma chloephagae* descripta en *Chloephaga picta leucoptera*, aunque las espinas de la corona en nuestros especímenes son más pequeñas. Comparando la presencia y los valores de infección de las metacercarias, *E. megacirrus* tiene un rango de distribución más amplio, se registra en más ambientes y presenta mayores prevalencias.

Palabras claves: Digenea, Hyriidae, metacercarias, infecciones experimentales, Sur de Argentina.

INTRODUCTION

Diplodon is endemic to the Neotropical Region and all species are distributed in freshwater environments in the Guayano-Brazilian sub-region (Castellanos and Landoni, 1995). In Argentina, there are 11 species, of which only *Diplodon chilensis* Gray (Hyriidae, Unioniformes) is present in the Patagonian Region (Rumi *et al*., 2008). It can been found on both sides of the Andes, from 30°28'S (River Grande, Coquimbo) to 51°50'S (Pond Diana, Puerto Natales, Magallanes) in Chile (Letelier, 2006; Parada *et al*., 2007) and from 38°55'S (Lake Aluminé, Neuquén) to 44°05'S (Lake Tres, Chubut) (Viozzi and Brugni, 2001) in Argentina. *Diplodon chilensis* is very abundant in lotic and lentic water bodies, living in silty and sandy-loam bottoms (Semenas and Brugni, 2002). This infaunal bivalve is dioicous, the fertilization of the females is performed in a special area of the gill, the marsupium, where the larval stage (glochidium) develops. The glochidia are expelled out parasitizing fins, tegument and gills of fish (Viozzi and Brugni, 2001; Brugni and Viozzi, 2002), and in Patagonia, infect both native and introduced fish (Semenas *et al*., 1994). The natural predators of this pelecypod are *Aegla abtao* Schmitt (pancora crab), *Podiceps major* (Boddaert) (huala) and *Lontra provocax* (Thomas) (huillín) (Lara and Moreno, 1995; Brugni and Viozzi, 2002).

Diplodon chilensis is important as a recycler of organic matter (Soto and Mena, 1999), can be used as a bio-indicator of contamination (Sabatini *et al*., 2011) and is also sensitive to competition from invasive species such as *Corbicula fluminea* (Müller) and *Limnoperna fortunei* (Dunker) (Torres *et al*., 2013). It is the most studied Hyriidae species of South America (Pereira *et al*., 2014) with numerous studies carried out in Chile and Argentina which include different aspects as ecological (Semenas and Brugni, 2002), conservational (Valdovinos and Pedreros, 2007), taxonomical (Parada and Peredo, 2008), morphological (Soldati *et al*., 2010), environmental (Sabatini *et al*., 2011), archaeological (Jackson and Jackson, 2012) and, physiological (Rochetta *et al*., 2014), among others. However, the knowledge about the role it plays as host for symbiotic and parasitic species is scarce.

The family Echinostomatidae is cosmopolitan and, the genera *Echinoparyphium* and *Echinostoma*, also are distributed in all continents and its definitive hosts may be birds or mammals (Yamaguti, 1971; Georgieva *et al*., 2014). Different authors suggest that an integration of the morphological and molecular information about species of these genera would be necessary to complete the knowledge of their diversity considering the recent descriptions of cryptic species in North America and Europe (Georgieva *et al*., 2014). Both genera are characterized by the presence of a crown of spines not interrupted dorsally. In the genus *Echinoparyphium*, the crown has two alternate rows with 29 to 45 spines of different size (Huffman and Fried, 2012). In South America, four species were registered, *Echinoparyphium singularis* (Lutz, 1924) with 33 spines parasitizing the pelecaniform *Tigrisoma* sp. from Brazil (Kohn and Fernandes, 1976), and the other three in Argentina, *Echinoparyphium recurvatum* (Linstow, 1873) in *Gallinula galeata galeata* (Lichtenstein) (Kohn and Fernandes, 1976), *Echinoparyphium scapteromae* Sutton 1983 in the rat of pajonal, *Scapteromys aquaticus* Thomas and in the common rat, *Rattus norvegicus* (Berkenhout) (Sutton and Lunaschi, 1994), and *Echinoparyphium megacirrus* Semenas, Brugni *et* Ostrowski de Núñez 1999 obtained experimentally in *Gallus gallus domesticus* from metacercariae collected from *D. chilensis* (Semenas *et al*., 1999). In the genus *Echinostoma*, the crown has 31 to 55 spines of equal size and their definitive hosts are birds and mammals (Kostadinova, 2005). In South America, 28 species have been recorded, 14 with 37 spines in the crown (Yamaguti, 1971; Kohn and Fernandes, 1975; Fernandes *et al*., 2015), from which *Echinostoma mendax* Dietz, 1909, *Echinosotma microrchis* Lutz, 1924, *Echinostoma equinatus gigas* Marco del Pont, 1926, *Echinostoma barbosai* Lie et Basch, 1966, *Echinostoma trivolvis* (Cort, 1914), *Echinostoma nephrocystis* Lutz, 1924, *Echinostoma neglectum* Lutz, 1924, *Echinostoma cloephagae* Sutton et Lunaschi 1980, parasitise only birds (Yamaguti, 1971; Kohn and Fernandes, 1975; Fernandes *et al*., 2015). Metacercariae of the latter genus, have been recorded in the bivalve *D. chilensis* (Semenas *et al*., 1999), in the snail *Chilina* spp. (Ostrowski de Núñez and Quaggiotto, 1995; Quaggiotto and Valverde, 1995; Flores, 2005)

and *Lymnaea viatrix* D'Orbigny (Prepelitchi, 2002) and were used for experimental infestations, but ovigerous adults were only obtained from the latter intermediate host.

In this scenery, the aim of this work was to evaluate the characteristics of infestations and the geographic distribution of *E. megacirrus* and *Echinostoma* sp. parasites of *D. chilensis* in Andean-Patagonian environments from southern Argentina and to enhance the knowledge of *Echinostoma* spp.

MATERIALS AND METHODS

The study area is located in Lanín, Nahuel Huapí and Los Alerces National Parks, which is characterized by a large hydrographic network that includes numerous ponds and, deep glacial ultra-oligotrophic or oligotrophic lakes.

Samplings were performed from December 2001 to March 2009, in 19 environments situated between 39°06'S and 42°36'S (Table 1). All specimens were collected manually at depths ranging from 0.50 to 1 m, transported live to the laboratory and maintained under controlled temperature conditions (6°C) until processed. Some specimens were used to study the characteristics of infestation in different environments and others, to perform experimental infestations (Table 1).

For studies of infestation characteristics, bivalves were measured (length in mm, maximum distance between the anterior and posterior margin of the valve), and the presence of metacercariae of the two species of Echinostomatidae was recorded. Prevalence was calculated for each site: overall (all the infected specimens), single (infected with any of the species of Echinostomatidae) and mixed infestations (infected with both species of Echinostomatidae). Some specimens of *D. chilensis* were fixed in buffered formalin for histological sections (7µm) and later, colored with hematoxylin and eosin.

For experimental infestations, parasitized viscera of *D. chilensis* were feed to *Gallus gallus domesticus* (chicks) of 3 days of age. In general, 1 chick per sample site was infected with an undetermined dose of metacercariae pools of *E. megacirrus* and *Echinostoma* sp. obtained from 10 to 20 specimens of *D. chilensis*. The experimental animals were maintained under appropriate conditions (food, water and, temperature) till were necropsied at different days postinfection (dpi). In some cases, metacercariae were isolated from viscera under a stereoscopic microscope, measured and mechanically crushed under coverslips to describe their morphological characteristics. The experimental adults obtained were stained and mounted for study under optical microscope for description and for recording maturity degree. Measurements of fixed specimens, colored with clorhidric carmine and mounted in Canada Balsam are given in micrometers and the range followed by

Table 1. Collection sites, coordinates, date, and number of specimens of *Diplodon chilensis* sampled in Patagonian freshwater environments (South Argentina).

Collection sites Lake (L) / Pond (P) / River (R)	Coordinates	Date	Number of sampled specimens
Pulmari (L)	39°06'S - 71°06'W	jan-2009	24
Ñorquinco (L)*	39°09'S - 71°17'W	mar-03	not recorded
Aluminé Pilolil (R)*	39°12'S - 71°12'W	mar-02	not recorded
Ruca Choroi (L)*	39°12'S - 71°12'W	feb- 02	not recorded
Lolog (L)	40°01'S - 71°24'W	dec-01	30
Machonico (L) *	40°21'S - 71°26'W	mar-02	not recorded
Espejo Chico (L)*	40°35'S - 71°44'W	feb-03	not recorded
La Larga (P)	40°42'S -71°00'W	feb-06	20
Mercedes (P)	40°42'S -71°00'W	feb-06	21
Piré (P)	40°43'S - 71°49'W	mar-09	22
Patagua (P) *	40°47'S - 71°37'W	feb-02	13
El Trébol (P)	41°04'S - 71°30'W	feb-02	30
Moreno Oeste (L) *	41°06'S - 71°32'W	mar-02	30
Los Césares (P) *	41°19'S - 71°43'W	feb-02	19
Mascardi (L) *	41°26'S - 71°64'W	mar-02 dec-03	64 29
Steffen (L) *	41°31'S - 71°35'W	feb-02	30
Puelo (L)*	42°07'S - 71°36'W	mar-02	not recorded
Epuyén (L)*	42°11'S - 71°30'W	feb-02	not recorded
Rivadavia (L) *	42°36'S - 71°39'W	mar-03	not recorded

* Specimens of *Diplodon chilensis* used for experimental infections

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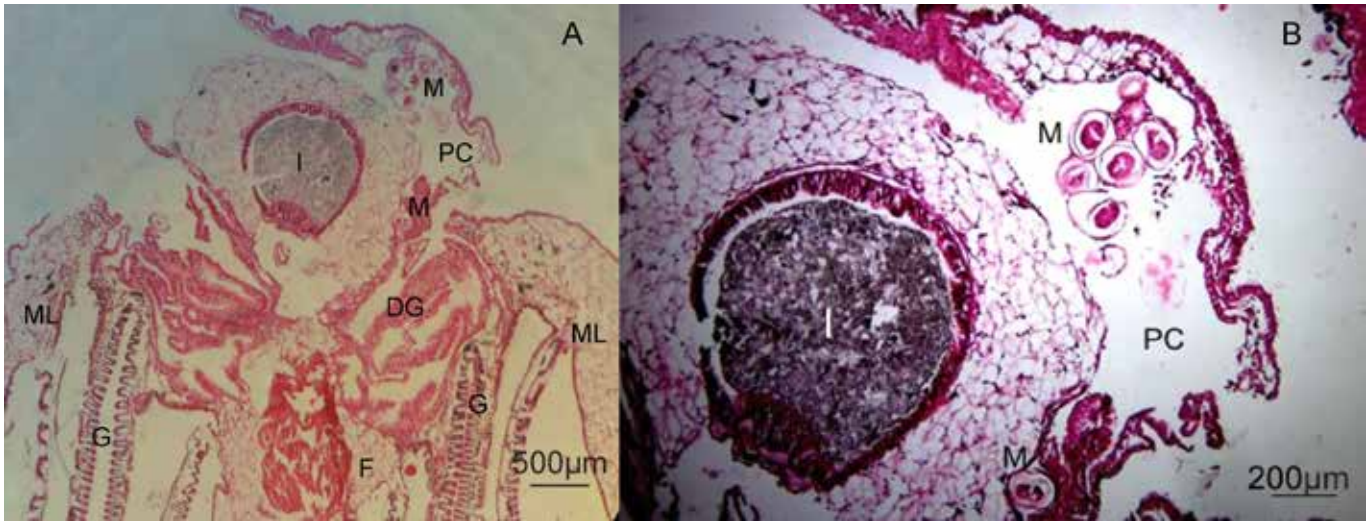

Figure 1. Transversal histological section of *Diplodon chilensis* infected with metacercariae. **A.** General view. **B.** Metacercariae in pericardial cavity. DG: digestive gland, F: foot; G: gill; I: intestine, M: metacercariae. ML: mantle lobule, PC: pericardial cavity.

mean and standard deviation in brackets. Only the measurements of specimens considered as "typical material", ie ovigerous adults of 13-14 days post infestation (d.p.i.) are included. The term "forebody" refers to the distance between the anterior border of body and the anterior edge of the ventral sucker, and the term "hindbody", to the distance between the posterior edge of the ventral sucker and the posterior border of the body. Comparisons of spines length were done with a Friedman test since samples are not independent and each individual represents a block (Conover, 1980).

Two paratypes of *E. chloephagae* from the Helminthological Collection of the Museum of La Plata (No. 582C) and 2 specimens of *Echinostoma* sp. from experimental infestations of *D. chilensis* from the Colección de Parasitología of the Centro Regional Bariloche, INIBIOMA (No. 46 1-2) were used for comparisons.

RESULTS

The specimens of *D. chilensis* collected in 19 environments had a length range between 48.4 and 85.2 mm (Table 2).

The metacercariae of Echinostomatidae are mainly located in the pericardial cavity and, also in hepatopancreas and gonads (Figure 1). Data of natural infestation of *D. chilensis* and of experimental infestation by metacercariae of Echinostomatidae indicates that the distribution range of *E. megacirrus* is wider, ranging from 39°06'S (Lake Pulmarí) to 42°36'S (Lake Rivadavia) than *Echinostoma* infestation, recorded from the 39°09'S (Lake Ñorquinco) to 41°31'S (Lake Steffen) (Tables 2 and 3). Additionaly, the presence of *E. megacirrus* is recorded in more environments (14 *versus* 8, Tables 2 and 3) and presents higher prevalence values (83.3 versus 27.3) (Table 2).

Adults, mainly ovigerous, were obtained from experimental infestations with metacercariae of *D. chilensis*. Ovigerous adults were obtained for *Echinostoma* in 5 localities, and in 6 for *E. megacirrus* (Table 3). Only in Mascardi and Steffen lakes, ovigerous specimens of both Echinostomatidae were obtained (Table 3). Ovigerous adults of *Echinostoma* were obtained for the first time from metacercariae of *D. chilensis*.

Echinopharyphium megacirrus: Metacercariae from *D. chilensis*

Localization: mainly pericardial cavity, also in hepatopancreas and gonads (Figure 1 A-B).

Measurements: Cyst 163-192 (169.1 ± 7.7) in diameter, whitish. Cyst wall thickness 2-10 (4.9 ± 1.3). Numerous calcareous corpuscles (about 100) with a diameter of 5-10 (6.5 ± 1.5). Collar with 43 spines: 4 angular, alternating two aborals and two orals, 4 lateral in single row and double row of 27 spines.

Experimental adults: (Figure 2 A - B)

Measurements (from 13 ovigerous experimental adults): Body 2,202-3,146 (2,604.8±331.8) long by 315-486 (398.0 ± 55.2) wide at level of ventral sucker, with parallel posterior margins of body, thinning up to the posterior area of the second testis. Forebody represents 17-26 (21.1±2.8)% of total length of body, and hindbody 61-72 (65.6±3.4)%. Tegument with scale shaped spines, 10-12 (11.8±0.75) long, in alternating and transverse rows, dorsally extend only to anterior edge of ventral sucker, lateral and ventrally to median line of ventral sucker. Cephalic collar well developed, 192-288 (238.3±29.7) wide with 43 spines: 4 angulars, 4 laterals in single row and double row of 27 spines (13 orals and 14 aborals). Alterning angular spines, aborals (2) and orals (2). Aboral spine I 43-54 (48.3±3.2), oral II, 52-67 (55.4±4.9), aboral III, 43-59 (51.3±5.1), and oral IV, 53-68 (58.4±5.4) long, with

significant differences among them ($\chi 2$=17.769; df=3, P=0.0001). The *a posteriori* test indicates medium length of spines in ascending order is aboral spine I, aboral III, oral II and, oral IV, all different between them. Four lateral spines: 43-50 (47.7±2.7); 43-52 (46.8±2.9); 44-52 (48.0±2.7), and 43-53 (48.0±3.5) long without significant differences between them ($\chi 2$=5.638; df=3, P=0.131). Oral spines 29-48 (39.6±5.7), and aboral spines 48-67 (57.3±5.2) long. Oral sucker subterminal 77-115 (95.3±9.9) long by 77-115 (93.8±11.2) wide. Oval ventral sucker situated between first and second fourth (second fifth), 259-384 (312.4±33.2) long by 221-346 (280.0±30.9) wide, ratio between sucker lengths 1: 2.8-4.4 (3.3±0.5). Pharynx 77-106 (86.4±7.1) long by 53-70 (61.1±6.2) wide. Esophagous 204-360 (304.8±59.2) long and caeca extends to posterior end of body.

Longitudinally elongated testes with smooth, contiguous margins, intercecal, in tandem occupying part of third and last fourth of body. Anterior testis 211-336 (284.3±35.8) long by 134-268 (183.2±35.1) wide, and posterior testis 288-403 (342.6±36.8) long by 106-211 (174.4±32.2) wide. Cirrus pouch elongated, posterior to caecal bifurcation, with muscular walls, overlapping in 43-59 (52.1±5.9)% of length with ventral sucker. Cirrus pouch 180-300 (248.8±33.4) long by 96-75 (131.6±27.3) wide, with simple seminal vesicle, pars-prostatica and, cirrus. Cirrus 264-576 (420.0±136.5) long by 36-38 (36.6±1.2) wide. Genital pore between bifurcation of caeca and anterior edge of ventral sucker. Ovary, spherical to transversely ovoid, in second fourth of body, situated at 154-403 (279.2±70.1) from ventral sucker, 96-173 (129.2±20.6) long by 115-154 (128.0±14.9) wide. Uterus intercaecal, and vitelline glands in lateral fields, anteriorly reaching posterior edge of ventral sucker, overlaps with margins of testes and converges in posterior testicular area. Vitelline ducts joint anteriorly to anterior testis forming a triangular reservoir. Yellowish eggs, 94-108 (97.1±4.4) long by 48-53 (50.2±2.1) wide. Excretory vesicle Y shaped.

***Echinostoma* sp.:**

Metacercariae from *D. chilensis*

Localization: mainly pericardial cavity, also in hepatopancreas and gonads (Figure 1 A-B).

Measurements: Cyst 182-202 (193.0±6.9) in diameter. Cyst wall thickness 10-14 (12.0±1.1). Numerous calcareous corpuscles (less than 100) with a diameter of 10-14 (12.0±1.1). Collar with 37 spines 10-19 (16.0±1.4) long: 5 angulars, from which 3 are orals and, 2 aborals, 6 laterals and, 15 dorsals, from which 8 are orals and, 7 aborals.

Experimental adults: (Figure 2 C-D)

Measurements (from 7 ovigerous experimental adults): Body 4,090-5,205 (4,804.8±394.2) long by 543-715 (608.8±75.2) wide at level of ventral sucker, with parallel posterior margins of body, and a slight constriction at posterior level of ventral sucker, posterior end slightly tilted. Forebody represents 12-16 (14.3±1.7)% of total body length, and hindbody 65-80 (75.9±5.1)%. Tegument with scale shaped spines, 10-14 (12.7±1.8) long, alternating in transverse rows, dorsally extending only to anterior edge of ventral sucker, ventrally beyond posterior testis, in this region are sharpened, laterally spines extends to posterior end of posterior testis. Well developed cephalic collar with 37 spines, representing the 6-7 (6.8±0.3)% of total body length, 298-355 (327.8±21.8) wide. Spines distributed in 5 corner spines (3 oral and, 2 aboral), 6 lateral, and 15 dorsal (8 oral and, 7 aboral). Corner spine I 31-53 (42.3±8.9) long, II 43-55 (49.8±4.9) long, III 35-53 (44.4±8.6) long, IV 44-65 (54.9±9.2)

Table 2. Collection sites, sample size, and length of specimens of *Diplodon chilensis* and prevalence of infection of metacercariae of Echinostomatidae.

Collection sites Lake (L) / Pond (P) / River (R)	Sample size	Mean length (Range)*	Prevalence (%)			
			Total	*Echinostoma* sp.	*Echinoparyphium megacirrus*	Both
Pulmari (L)	24	65.87 (38.07- 88.2)	29.2	0	29.2	0
Lolog (L)	30	67.8 (53.1-78.7)	70	0	66.6	3.4
Piré (P)	22	77.1 (57.9-102.5)	68.2	27.3	0	40.9
Patagua (P)	13	48.4 (28.6-60.1)	46.2	0	0	46.2
La Larga (P)	20	54.2 (25.4-72.4)	30	0	30	0
Mercedes (P)	21	53.3 (36.6-65.9)	61.9	0	61.9	0
El Trébol (P)	30	55.7 (28.7-85.9)	3.3	0	3.3	0
Moreno Oeste (L)	30	62.4 (32.9-88)	43.3	0	30	13.3
Los Césares (P)	19	85.2 (67.4-109.5)	10.5	0	0	10.5
Mascardi (L)	29	76.9 (70.3-89.4)	48.3	3.5	37.9	6.8
Steffen (L)	30	85.1 (70.6-98)	90	0	83.3	6.6

* Measurements in mm

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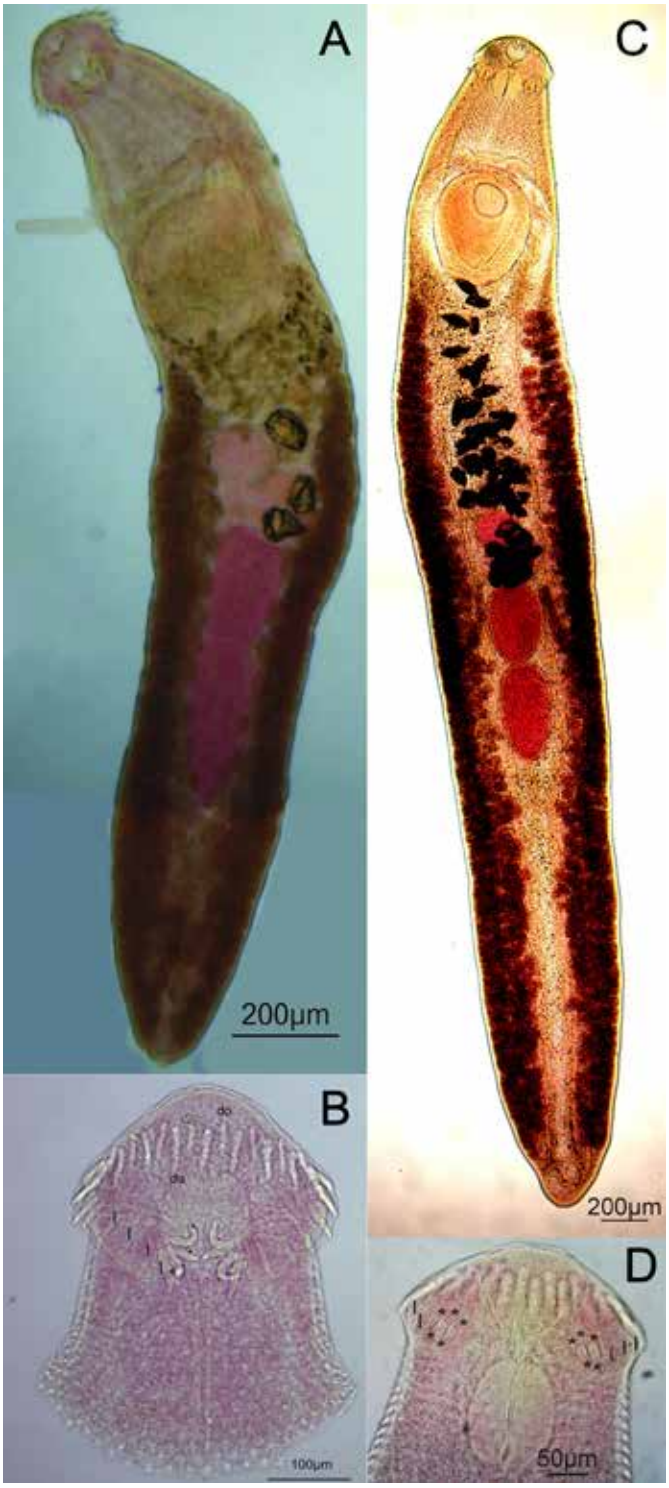

Figure 2. Microphotographies of experimental adults of Echinostomatidae obtained from metacercariae of *Diplodon chilensis*: **A.** Adult of 7 days post infection of *Echinoparyphium megacirrus*, **B.** Collar spines of adult of *Echinoparyphium megacirrus*, **C.** Adult of 14 days post infection of *Echinostoma* sp., **D.** Collar spines of adult of *Echinostoma* sp. *****: corner spines; **l**: lateral spines; **da**: dorsal aboral spines; **do**: dorsal oral spines.

long, and V 38-53 (45.6±6.0) long. The lateral I 43-60 (52.8±7.6) long, II 44-66 (55.5±8.9) long, III 44-65 (54.6±8.3) long, IV 44-55 (50.8±5.7) long, V 44-53 (49.2±4.3) long, and VI 48-53 (50.4±3.4) long. Oral spines of dorsal row 41-62 (48.6±9.7) long, and aboral 53-60 (56.4±4.2) long. Spines have significant differences in length among them ($\chi 2=24.483$; df=3; P= 0.004). The *a posteriori* test indicates that angular spines I to III and, oral ones are similar between them, and angular IV, lateral I and II, and aborals are similar between them. Oral sucker ventro-subterminal, 115-163 (141.3±24.6) long by 115-154 (134.4±18.4) wide. Ventral sucker slightly oval, 384-538 (464.9±71.2) long by 384-480 (423.8±40.9) wide situated in first fourth in ovigerous specimens, and in 83.3% of immature specimens situated between first and, second fourth of body. Ratio between sucker lengths 1:3-4 (3.3±0.4). Prepharynx short, sometimes indistinguishable, 0-41 (22.3 ± 16.3) long. Oval pharynx 120-156 (136.8±13.6) long by 103-149 (120.7±15.3) wide. Ratio between length of oral sucker and pharynx 1: 1 (1.0±0.1). Esophagous 240-312 (274.0±30.8) long, representing the 5-7 (5.8±0.8)% of body length. Caeca extends to posterior end of body.

Longitudinally elongated testes with smooth margins, intercecal, in tandem, occupying third fourth of body in immature and ovigerous specimens. Intertesticular distance 0-50 (18.4±24.0). Anterior testis situated 672-1,574 (1,322.1±308.9) from ventral sucker, 202-384 (318.2±61.8) long by 173-307 (220.8±46.4) wide; posterior testis 298-432 (364.8±53.7) long by 182-259 (215.3±28.2) wide, ratio length-width 1:1.3-1.8. Situated at 1,277-1,949 (1,627.9±279.3) from posterior body border, representing 38 (33.7±4.0)% of total body length. Cirrus pouch elongated, posterior to caecal bifurcation, 312-432 (394.3±49.7) long by 216-300 (257.1±33.8) wide, overlapping in 80-99 (85.3±7.1)% with ventral sucker length, contains simple and oval seminal vesicle, pars-prostatica and cirrus without spines. Cirrus (n=1) 216 long. Genital pore between bifurcation of caeca and anterior end of ventral sucker. Ovary, spherical to transversely ovoid, 96-240 (164.6±44.1) long by 106-259 (167.3±52.2) wide, situated in second fourth in 95% of ovigerous specimens, and in third fourth in 90% of immature specimens, situated at 960-1,229 (1,119.1±101.3) from ventral sucker, and at 115-240 (178.3±41.4) from anterior testis. Uterus intercaecal with numerous coils, situated between ventral sucker and ovary, with distal metraterm opening in a common atrium. Vitelline glands in lateral fields, slightly overlaping caeca, not confluent. Vitelline ducts joint anteriorly to the anterior testis forming a triangular reservoir. Yellowish eggs 98-120 (113.3±9.8) long by 50 wide, egg number 4-100 (47.3±31.6). Excretory vesicle Y shaped bifurcating behind the posterior testis and opening at the end of the body through a pore.

DISCUSSION

In Patagonia, infestations with different genera of the family Echinostomatidae have been reported in secondary intermediate hosts such as the flatworm

Temnocephala chilensis Moquin-Tandom, the snails *Chilina* spp., and *L. viatrix*; and the bivalve *D. chilensis* in Neuquén, Río Negro and Chubut Provinces (Ostrowski de Núñez and Quaggiotto, 1995; Quaggiotto and Valverde 1995; Semenas *et al.*, 1999; Flores, 2005; Viozzi *et al*., 2005; Prepelitchi and Ostrowski de Núñez, 2007). The present study has allowed not only to enlarge the range of distribution in Patagonia of 2 species of the family Echinostomatidae, *E. megacirrus* and *Echinostoma* sp, but also determine that *E. megacirrus* has a wider distribution, is present in more environments, and has higher prevalence values than *Echinostoma* sp. (see Table 2). The only previous values of prevalence of metacercariae of *E. megacirrus* were from Lake Gutiérrez (Semenas *et al*., 1999), and are similar to our values.

The measurements of both, metacercariae and experimental ovigerous adults, of *E. megacirrus* agree with measurements given in the original description of Semenas *et al*. (1999), so they were assigned to this species without any doubt.

The metacercariae of *Echinostoma* sp. obtained, have a smaller diameter of the cyst and a greater thickness of the wall, a smaller size of the crown of spines, and differences in the distribution of spines in comparison to the description in Semenas *et al*. (1999). The distribution of the angular and lateral spines is 5-5-6-6 (Figure 2D) whereas in the previous description it is 4-4-5-5. The review of 2 specimens deposited in the Colección de Parasitología of the Centro Regional Bariloche (No. 46/1-2) did not help to solve this discrepancy.

The experimentally obtained ovigerous adults of *Echinostoma* sp. correspond to the group "*revolutum*" characterized by a collar of 37 spines. The species differentiation of this group is complex given their morphological similarities, nomenclature problems caused by inadequate descriptions, poor diagnoses, extensive synonyms as well as inaccessibility and loss of type material (Kostadinova and Gibson, 2000). Our recovered adult specimens resemble to species with smooth margins of testes, according to the key of "*revolutum*" group of the European species (Georgieva *et al*., 2014). However, their body size is smaller (maximum length 6,000 µm *versus* European 9,000 µm), length range of collar spines is intermediate (43-53 µm), and egg length (98-120 µm *versus* "less than 105 µm or greater than 130 µm") is not within the range indicated in the key (Faltýnková *et al*., 2015). Our specimens have a collar diameter less than 500 µm and the maximum width of the body located at level of ventral sucker, similar to *Echinostoma bolschewense* (Kotova, 1939), but differs from this species by the ratio of testes length to width (1: 1.3-1.8 *versus* 1:2.30-2.33), and by egg length (98-120 µm *versus* 138-162 µm) (Faltýnková *et al*., 2015). In Southern Hemisphere, one species with a collar of 37 spines was described from Africa: *Echinostoma deserticum* Kechemir, Jourdane *et* Mas-Coma 2010, which differs from our specimens by larger body size (up to 15,000 µm), maximum body width beyond ventral sucker, variable number of testes (0 to 2) and smaller eggs

Table 3. Data of experimental infections with metacercariae of *Echinostoma* sp. and *Echinoparyphium megacirrus* obtained from *Diplodon chilensis*.

Collection sites Lake (L) / Pond (P) / River (R)	Post-infection days	*Echinostoma* sp.		*Echinoparyphium megacirrus*	
		Number of adults obtained	Maturity degree	Number of adults obtained	Maturity degree
Ñorquinco (L)	14	2	ovígerous	-	-
Aluminé Pilolil (R)	14	0	-	-	-
Ruca Choroi (L)	14	0	-	-	-
Machonico (L)	14	0	-	-	-
Patagua (P)	7	0	-	329	ovígerous
Espejo Chico (L)	7	0	-	4	ovígerous
	14	0	-	-	-
Moreno Oeste (L)	13	1	ovígerous	-	-
	21	0	-	-	-
Mascardi (L)	13	36	ovígerous/ inmature	6	ovígerous
	7	1	inmature		
Los Césares (P)	8	18	inmature	-	-
	13	1	ovígerous	-	-
Steffen (L)	13	1	ovígerous	5	ovígerous
Puelo (L)	14	0	-	52	ovígerous
Epuyén (L)	14	0	-	-	-
Rivadavia (L)	14	0	-	90	ovígerous

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(58-74 µm by 36-46 µm) (Kechemir *et al.*, 2010).

The species of *Echinostoma* with collar of 37 spines described for birds in South America are: *E. mendax*, *E. microrchis, E. nephrocystis*, *E. equinatus gigas*, *E. barbosai*, *E trivolvis*, *E. neglectum,* and *E. chloephagae.* Our specimens differ from *E. mendax* in a smaller body (up to 9,500 µm long), bigger eggs (98-120 µm long by 50 µm wide *versus* 90-120 µm, 58-62 µm) and by infestation site in their avian hosts (small intestine *versus* rectum) (Boero *et al*., 1972; Fernandes *et al*., 2015). Although *E. microrchis* and our specimens have a similar body size, they differ in egg size which are smaller in *E. microrchis* (84-93 µm by 47-56 µm), and in distribution of vitelline glands, being confluent in the former (Kohn and Fernandes, 1975; Fernandes *et al.*, 2015), while in our specimens are not confluent. Our specimens differ from *E. nephrocystis,* in the body shape which presents its maximum width between ovary and testes, while in ours the maximum is at the level of ventral sucker, and testes transversely elongated (Kohn and Fernandes, 1975) *versus* longitudinally elongated in ours. The specimens described herein differ from *E. equinatus gigas* by having a smaller body size (4-5 by 0.5-0.7 mm *versus* 35-40 by 3-4 mm), and indented/lobed testis (see Figure 7, in Marco del Pont, 1926) instead of smooth ones; from *E. barbosai* by a bigger metacercariae cyst (182- 202 µm *versus* 145-158 µm), smaller maximum length of collar spines (66 µm *versus* 89 µm) and, smaller eggs (98-120 µm long by 50 µm wide *versus* 111-131 µm by 62-66 µm) (Lie and Basch, 1966). The experimental adults obtained from *D. chilensis* differ from *E. trivolvis* by a bigger metacercariae cyst (182- 202 µm *versus* 135-178 µm), smaller body length (4,090-5,205 µm *versus* 5,500-21,000 µm), maximum length of collar spines (66 µm *versus* 120 µm), and smaller eggs (98-120 µm long by 50 µm wide *versus* 90-130 µm by 60-70 µm) (Kanev *et al*., 1995), and from *E. neglectum* by having larger eggs (98-120 µm long by 50 µm wide *versus* 75-105 µm by 38-64 µm), and smaller ventral sucker (384-538 µm *versus* 500-720 µm) (Kohn and Fernandes, 1975).

The comparison with the natural specimens of *E. chloephagae*, described from the native anatid *Chloephaga picta leucoptera* from the Malvinas islands (Sutton and Lunaschi, 1980), indicates that morphological characteristics and measurements are within the range described of our experimental specimens. The collar spines have the same distribution but spines are larger in our specimens (64-84 *versus* 31-65 µm), which could be due to age differences between natural and experimental specimens. These similarities would indicate that our specimens belong to *E. chloephagae*, Prepelitchi (2002) also indicates that the experimental ovigerous adults obtained from *L. viatrix* could correspond to *E. chloephagae*. Additionally, the morphology and the measurements of these experimental adults are similar to ours.

In the present study we obtained experimentally ovigerous specimens of *Echinostoma* from metacercariae of *D. chilensis*. In the previous experimental infestations performed by Semenas *et al.* (1999) specimens did not mature, probably because the infestation was done separating metacercariae of the two echinostomid species. Prepelitchi (2002) obtained similar results, in mixed and single infestations with metacercariae of *Echinoparyphium* spp. and *Echinostoma* sp., observing differences in growth and maturation of the second species in presence of any *Echinoparyphium* species. To our knowledge only site segregation experimental studies between *Echinostoma* and *Echinoparyphium* have been performed (Baruš *et al*., 1974), but none study, except Prepilitchi´s (2002), was done over the effect on growth and maturation. Although, more experimental studies must be performed to elucidate the interactions on growth and maturation when species of *Echinostoma* and *Echinoparyphium* co-exist in the same host.

About the potential life cycle of *E. chloephagae*, it would include as first intermediate host *Chilina* spp., and *L viatrix* (Ostrowski de Núñez and Quaggiotto, 1995; Quaggiotto and Valverde, 1995; Prepilitchi, 2002; Flores, 2005); and as second intermediate host *Chilina* spp., *L viatrix* and, *D. chilensis* (Quaggiotto and Valverde, 1995; Semenas *et al.,* 1999; Prepilitchi, 2002; Flores, 2005). Adults of *E. chlophagae* were described from *C. picta leucoptera*, but considering their diet, perhaps infestation may be accidentally acquired foraging on pastures snails like *L. viatrix*. Except *Chloephaga melanoptera* (Eyton), any of the other four species of *Chloephaga* could be definitive host considering their migratory habits (Pedrana *et al*., 2011; 2015), and their distribution in continental Patagonia and Malvinas Islands (Narosky e Yzurieta, 2010). For *E. megacirrus,* the potential hosts could include *Chilina* spp. as first intermediate host (Ostrowski de Núñez and Quaggiotto, 1995; Quaggiotto and Valverde, 1995; Flores, 2005) and *Chilina* spp., *D. chilensis* and, *T. chilensis* as second ones (Semenas *et al.,* 1999; Flores, 2005; Viozzi *et al*., 2005). Up to now no natural infections have been recorded for definitive hosts.

Echinostoma and *Echinopharyum* species are not specific for their definitive host, which may be birds and mammals, so *P. major* and *L. provocax* could also be definitive hosts in Patagonian environments, because they include *D. chilensis* in their diets. Although, adults of *E. megacirrus* and *Echinostoma* sp. were obtained from experimental infestations in chickens (Semenas *et al*., 1999; this study); those in mice were

unsuccessful; probably post infestation days were not enough to allow specimens to mature (4-5 dpi, see Semenas *et al.,* 1999).

This study let enlarge known distribution of *E. megacirrus* and *Echinostoma* in Patagonian freshwater environments, add ecological data about prevalence of infestations and, include *D. chilensis* as a new second intermediate host for *Echinostoma* sp.

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