Anthropozoonotic Parasites Circulating in Synanthropic and Pacific Colonies of South American Sea Lions (*Otaria flavescens*): Non-invasive Techniques Data and a Review of the Literature

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Since late 1970s, the southern Chilean city Valdivia constitutes home for a unique bachelor group of South American sea lions (*Otaria flavescens*), initially descendant from colonies at the Pacific coast, but now directly living in a freshwater habitat in close proximity to human population and a vast amount of wild and domestic animal species. In the framework of a parasitological monitoring program, 115 individual fecal samples were collected from synanthropic South American sea lions between March and May 2018. For comparative reasons, 79 individual fecal samples from two free-living *O. flavescens* colonies at the Pacific coast were also sampled. Coproscopical analyses revealed the presence of nine different parasite taxa in individual fecal samples, including two protozoan (*Cryptosporidium* spp. and *Giardia* spp.) and seven metazoan parasites (Anisakidae gen. spp., Diphyllobothriidae gen. spp., *Ogmogaster heptalineatus*, Trematoda indet. type 1, Trematoda indet. type 2, *Otostrongylus circumlitus*, and *Parafilaroides* spp.), and morphological and molecular characterizations of adult helminths confirmed identification of following species: *Anisakis simplex/A. pegreffi*, *Pseudoterranova cattani*, *Contracaecum ogmorhini*, and *Adenocephalus pacificus*. For the first time, the results of the current study show the presence of zoonotic relevant *Giardia*- and *Cryptosporidium*-infections in two free-ranging colonies of South American sea lions apart from human settlement. Furthermore, a detailed literature search of previous publications on the endoparasite fauna of South American sea lions was conducted, revealing reports of at least 50 protozoan and metazoan parasite taxa including findings of the current study. Thereby, at least 25 of reported taxa (50%) have been recorded to bear zoonotic potential. The

present study illustrates a successful application of non-invasive screening methods and their applicability in the field of marine mammal parasitology, bringing new insights into the endogenous parasite fauna of South American sea lions in Southern Chile, including anthropozoonotic protozoan and metazoan taxa.

INTRODUCTION

The South American sea lion (*Otaria flavescens*) constitutes a representative of the pinniped family Otariidae, the eared seals, and shows a broad geographical extension among Pacific and Atlantic coastlines of South America, including Peru, Chile, Argentina, Uruguay, Southern Brazil and the Falkland Islands (Vaz-Ferreira, 1982). Representing a polygynous species, *O. flavescens* males can lead harem structures or live together in bachelor groups, which usually occur at the edge of breeding colonies (Cárdenas-Alayza, 2017). Studies on movement patterns of *O. flavescens* males and females revealed a maximum travel distance of more than 850 km per trip (Campagna et al., 2001), even though South American sea lions do not show a real migratory behavior (Vaz-Ferreira, 1982). Interactions and conflicts with humans were described to mainly occur around salmon breeding farms (Sepúlveda and Oliva, 2005) or during industrial fishery, where sea lions compete with fishing activities trying to prey out of trawling nets (Szteren and Páez, 2002; Hückstädt and Antezana, 2003; De María et al., 2014). Thus, some studies documented South American sea lions as bycatches (Majluf et al., 2002; Reyes et al., 2013; Machado et al., 2015). According to International Union for Conservation of Nature (IUCN), *O. flavescens* is currently classified as a species of least concern (Cárdenas-Alayza et al., 2016).

Wild sea lion species have previously been reported to adapt to synanthropic lifestyles, e.g., the Galápagos sea lions (*Zalophus wollebaeki*) in Ecuador (Denkinger et al., 2015), or the Californian sea lions (*Z. californianus*) at Pier 39 in San Francisco, United States (Heath and Perrin, 2009). Colonies of free-ranging South American sea lions have been also consistently reported to adapt to human populations in the Chilean harbor cities of Valparaíso, Tomé and Talcahuano. In contrast to former colonies, the city of Valdivia harbors the only synanthropic South American sea lion colony worldwide, constantly living in a freshwater habitat 15 km east of the Pacific Ocean since late 1970s (Schlatter, 1976). Initially descendant from colonies at the Pacific coast, approximately 70 adult South American sea lion males are currently living in this anthropogenic ecosystem in closeness to humans and numerous domestic and wild endemic animals, including stray dogs (*Canis lupus familiaris*), cats (*Felis silvestris catus*), water birds [i.e., Peruvian pelicans (*Pelecanus thagus*), cormorants (*Phalacrocorax brasilianus*) and kelp gulls (*Larus dominicanus*)], turkey (*Carthartes aura*), and black vultures (*Coragyps atratus*).

Synanthropic South American sea lions of Valdivia differ from other free-ranging populations at Pacific coast mainly by their adaptation to anthropogenic stress, including boating-, shipping- and other sport-related activities and traffic on the river Calle-Calle. They also display altered feeding habits. Interspecific interactions occur on a daily basis and are decisive for maintenance of diverse endo- and ectoparasite life cycles in this urban colony (Sepúlveda et al., 2015; Hermosilla et al., 2016a; Cornejo-Galaz, 2017; Ebmer et al., 2019).

Most of the previous studies on the endogenous parasite fauna of free-living *O. flavescens* focused on helminths and were mainly based on single species reports of nematodes (Baylis, 1933; Carrara, 1952; López-Fernández, 1967; Botto and Mañé-Garzón, 1975; Cattan et al., 1976; Cattan and Carvajal, 1980; George-Nascimento et al., 1992; George-Nascimento and Llanos, 1995; George-Nascimento and Urrutia, 2000; Berón-Vera et al., 2004; Nadler et al., 2013; Timi et al., 2014; Jacobus et al., 2016; Machado-Pereira et al., 2017; González et al., 2018; Pasqualetti et al., 2018), cestodes (Markowski, 1952; Miranda et al., 1968; Cattan et al., 1977; Mondragón-Martínez, 2017), trematodes (Petrov and Chertkova, 1963; Carvajal et al., 1983; Hernández-Orts et al., 2012), and acanthocephalans (Morini and Boero, 1960; Zdzitowiecki, 1986; George-Nascimento and Marin, 1992; Aznar et al., 2006, 2012; Hernández-Orts et al., 2017, 2019). Moreover, several large-scale examinations were also published (Baylis, 1934; Holcman-Spector et al., 1977; George-Nascimento and Carvajal, 1980, 1981; Fernández, 1987; Cabrera et al., 1994; Andrade et al., 1998; Morgades et al., 2006; Pereira, 2012; Hernández-Orts et al., 2013b; Pereira et al., 2013; Calderón-Mayo, 2015; Sepúlveda et al., 2015; Seguel et al., 2018; Naupay et al., 2019) (**Table 1**). However, the vast majority of these studies were carried out during pathological dissections of stranded animals. Considering conservation aspects, we recently published two non-invasive fecal sample monitoring studies on parasites of South American sea lions, detecting also zoonotic relevant protozoan parasites such as *Giardia*, *Cryptosporidium*, *Neobalantidium*, and *Entameba* for first time (Hermosilla et al., 2016a, 2018b). Up to date, knowledge on protozoan parasites infecting urban and Pacific South American sea lions remains very scarce. Furthermore, detection of adult helminths and lungworms constitutes a major challenge in the field of non-invasive wildlife parasitology. Hermosilla et al. (2018b) already showed the possibility of pronounced morphological and molecular analyses of spontaneously excreted cestodes and mentioned the importance of expansion of these sampling techniques to extend knowledge on the parasite fauna of these animals.

TABLE 1 | List of endoparasites reported from South Americans sea lions (*Otaria flavescens*), including findings of present study.

Parasite	Localization	Zoonotic potential	Literature
Protozoa			
Toxoplasma gondii[a]	Chile	Yes	Sepúlveda et al. (2015)
Cryptosporidium spp.	Chile	Yes	Hermosilla et al. (2016a), Cornejo-Galaz (2017); Present study
Entamoeba spp.	Chile	Yes	Hermosilla et al. (2018b)
Giardia spp.	Chile	Yes	Hermosilla et al. (2016a), Present study
Isospora spp.	Chile	No	Hermosilla et al. (2016a)
Neobalantidium spp.	Chile	Yes	Hermosilla et al. (2016a, 2018b)
Metazoa			
Acanthocephala			
Bolbosoma turbinella	Brazil	Unknown	Pereira (2012)
Corynosoma australe (syn. *Corynosoma otariae*)	Argentina, Brazil, Peru, Uruguay	Unknown	Morini and Boero (1960), Andrade et al. (1998), Aznar et al. (2006, 2012), Morgades et al. (2006), Pereira (2012), Hernández-Orts et al. (2013b; 2017; 2019), Calderón-Mayo (2015), Naupay et al. (2019)
Corynosoma cetaceum	Argentina	Unknown	Hernández-Orts et al. (2013b)
Corynosoma gibsoni	Falkland Islands	Unknown	Zdzitowiecki (1986)
Corynosoma obtuscens	Peru, Uruguay	Unknown	Miranda et al. (1968), Holcman-Spector et al. (1977), Tantaleán et al. (2005)
Profilicollis chasmagnathi	Argentina	Unknown	Hernández-Orts et al. (2013b)
Andracantha sp.	Argentina	No	Hernández-Orts et al. (2013b)
Bolbosoma sp.	Peru, Uruguay	Yes	Holcman-Spector et al. (1977), Calderón-Mayo (2015)
Corynosoma sp./spp.	Brazil, Chile, Uruguay	Yes	Baylis (1934), George-Nascimento and Carvajal (1981), Fernández (1987), George-Nascimento and Marin (1992), Morgades et al. (2006), Jacobus et al. (2016)
Cestoda			
Adenocephalus pacificus (syn. *Diphyllobothrium pacificum*)	Chile, Falkland Islands, Peru	Yes	Baylis (1934), Markowski (1952), Miranda et al. (1968), Cattan et al. (1977), George-Nascimento and Carvajal (1981), Fernández (1987), Cabrera et al. (1994), Mondragón-Martínez (2017), Naupay et al. (2019), Present study
Diphyllobothrium arctocephalinum	Peru	Yes	Calderón-Mayo (2015)
Phyllobothrium delphini[b]	Chile	No	George-Nascimento and Carvajal (1981)
Anophryocephalus sp.	Chile	No	Fernández (1987)
Diphyllobothrium sp./spp.	Argentina, Brazil, Chile, Uruguay	Yes	Morgades et al. (2006), Pereira (2012), Hernández-Orts et al. (2013b), Sepúlveda et al. (2015), Hermosilla et al. (2018b)
Phyllobothrium sp.	Argentina, Falkland Islands	No	Baylis (1934), Carrara (1952)
Diphyllobothriidae gen. sp./spp.	Chile	Yes	Hermosilla et al. (2016a, 2018b), Present study
Nematoda			
Anisakis simplex[c]	Chile	Yes	George-Nascimento and Carvajal (1981), Fernández (1987), Present study
Anisakis pegreffi[c]	Chile	Yes	Present study
Contracaecum ogmorhini	Argentina, Brazil, Chile	Yes	Hernández-Orts et al. (2013b), Machado-Pereira et al. (2017), Present study
Contracaecum osculatum	Argentina, Chile, Peru	Yes	Carrara (1952), Miranda et al. (1968), Fernández (1987), Calderón-Mayo (2015), Seguel et al. (2018), Naupay et al. (2019)
Contracaecum rectangulum	Falkland Islands	Yes	Baylis (1934)
Pseudoterranova cattani[d]	Argentina, Chile	Yes	Cattan and Carvajal (1980), George-Nascimento and Carvajal (1980), George-Nascimento and Carvajal (1981), Fernández (1987), George-Nascimento and Urrutia (2000), Hernández-Orts et al. (2013b), Timi et al. (2014), Present study
Otostrongylus circumlitus	Chile	No	Present study
Trichinella spiralis	Argentina	Yes	Pasqualetti et al. (2018)
Uncinaria hamiltoni	Argentina	Yes	Baylis (1933); Berón-Vera et al. (2004), Hernández-Orts et al. (2013b), Nadler et al. (2013), González et al. (2018)
Uncinaria hamiltoni platensis	Uruguay	Yes	Botto and Mañé-Garzón (1975), Holcman-Spector et al. (1977)
Anisakis sp./spp.	Argentina, Chile	Yes	Cattan et al. (1976), George-Nascimento and Carvajal (1980), Hernández-Orts et al. (2013b), Hermosilla et al. (2018b)
Contracaecum sp./spp.	Argentina, Brazil, Chile, Uruguay	Yes	López-Fernández (1967), George-Nascimento and Carvajal (1980) George-Nascimento and Carvajal (1981), Andrade et al. (1998), Morgades et al. (2006), Hernández-Orts et al. (2013b), Timi et al. (2014), Sepúlveda et al. (2015)

(Continued)

TABLE 1 | Continued

Parasite	Localization	Zoonotic potential	Literature
Otostrongylus sp.	Chile	No	Hermosilla et al. (2016a)
Parafilaroides spp.	Chile	No	Hermosilla et al. (2018b), Present study
Pseudoterranova sp. (syn. *Phocanema* sp.)	Chile	Yes	Cattan et al. (1976), George-Nascimento and Llanos (1995), Sepúlveda et al. (2015)
Uncinaria sp.	Chile, Peru, Uruguay	Yes	George-Nascimento et al. (1992), Morgades et al. (2006), Calderón-Mayo (2015), González et al. (2018)
Anisakidae gen. sp./spp.	Chile, Peru, Uruguay	Yes	Cabrera et al. (1994), Morgades et al. (2006), Hermosilla et al. (2016a), Present study
Metastrongylidae gen. spp.	Peru	No	Gonzales-Viera et al. (2011)
Trematoda			
Ascocotyle (Phagicola) longa	Brazil, Uruguay	No	Morgades et al. (2006), Pereira et al. (2013)
Ascocotyle (Ascocotyle) patagoniensis	Argentina	No	Hernández-Orts et al. (2012, 2013b)
Ogmogaster heptalineatus	Chile, Peru	No	Carvajal et al. (1983), Fernández (1987), Calderón-Mayo (2015), Sepúlveda et al. (2015), Naupay et al. (2019), Present study
Stephanoprora uruguayense	Brazil	No	Pereira et al. (2013)
Zalophotrema lubimowi[e]	Russia	No	Petrov and Chertkova (1963)
Lobatostoma sp.[f]	Peru	No	Calderón-Mayo (2015)
Ogmogaster sp.[g]	Chile	No	George-Nascimento and Carvajal (1981)
Stephanoprora sp.	Uruguay	No	Morgades et al. (2006)
Trematoda indet. type 1	Chile	Unknown	Hermosilla et al. (2016a), Present study
Trematoda indet. type 2	Chile	Unknown	Present study

[a]Antibodies against T. gondii were detected.
[b]Plerocercoids reported in the abdominal wall.
[c]Differentiation between A. simplex s.s. and A. pegreffi was not possible within this study.
[d]Pseudoterranova cattani was first described by George-Nascimento and Urrutia (2000), and reported as Phocanema decipiens and Pseudoterranova decipiens in Chile in the past.
[e]Only one report from O. flavescens in captivity.
[f]Potentially fish parasite detected after gastrointestinal passage.
[g]Two years later, Carvajal et al. (1983) described O. heptalineatus from the same region.

In the framework of One Health, the present study aimed to investigate the endogenous parasite fauna of a synanthropic South American sea lion colony with special emphasis on anthropozoonotic parasitoses and compared them to parasites circulating in *O. flavescens* colonies occurring off the Pacific coast. In order to give an overview of the current scientific knowledge of the endoparasite fauna of *O. flavescens*, a detailed literature search was conducted and previously published reports were listed.
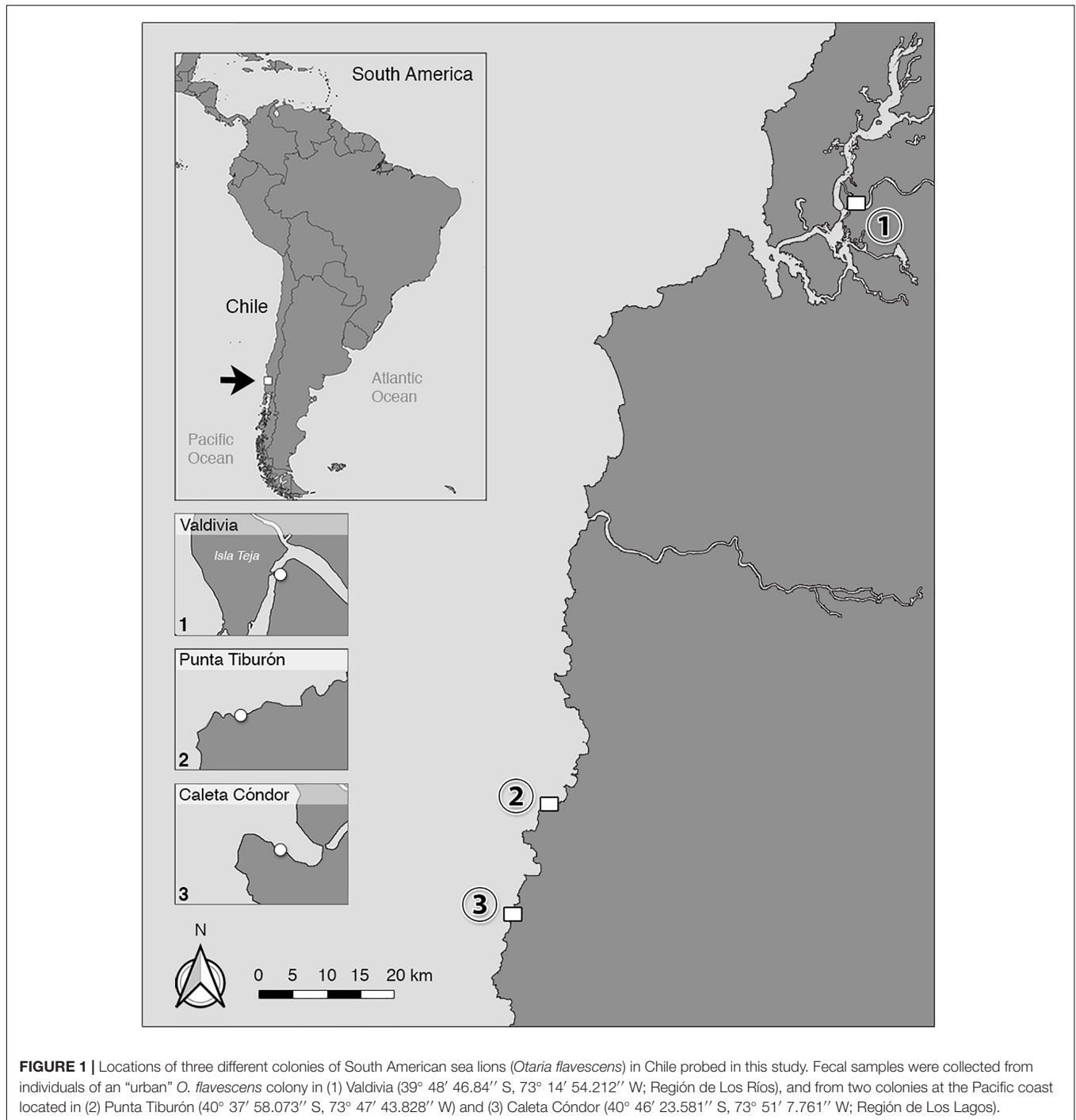
MATERIALS AND METHODS

Study Areas and Sample Collection

Between March and May 2018, three different free-ranging colonies of South American sea lions (*O. flavescens*) were investigated in Southern Chile to gain data on their endoparasite fauna. In the city of Valdivia (39° 48′ 46.84′′ S, 73° 14 54.212′′ W; 14th region of Chile: Región de Los Ríos) a special synanthropic colony of South American sea lion males living along freshwater river Calle-Calle (**Figures 1**, **2**) was studied representing a continuation of the previous parasitological monitoring with focus on anthropozoonotic parasite species (Hermosilla et al., 2016a). Sample collection was carried out through non-invasive

fecal sample monitoring following recent studies that utilized a stress-reduced methodology in parasitological research of sea lions (Hermosilla et al., 2016a, 2018b). In total, 115 individual fecal samples were collected at resting places frequented by South American sea lions ashore, such as construction areas (former riverside dock), the local fish market, and floating platforms on the river Calle-Calle.

Wild *O. flavescens* colonies allocated in the Province of Osorno, Chile (10th region: Región de Los Lagos) were further sampled within this study and chosen as comparison group. Therefore, a boat trip to two different *O. flavescens* colonies was conducted and scat samples were collected near Punta Tiburón (*n* = 38 individual samples; 40° 37′ 58.073′′ S, 73° 47′43.828′′ W) and Caleta Cóndor (*n* = 41 individual samples; 40° 46′ 23.581′′ S, 73° 51′ 7.761′′ W), both located approximately 120 km south of the city of Valdivia. Both sea lion colonies consisted mainly of females and juveniles and were traced and observed in rugged shores using a camera drone (DJI Mavic Pro®, Shenzhen, China). When we entered the cliffs, some animals immediately escaped into the water but others (mainly adult males) remained on the rocks.

Whenever possible, excrements of urban sea lions were immediately collected after spontaneous defecation and registered to individual animals. In general, only well defined

FIGURE 1 | Locations of three different colonies of South American sea lions (*Otaria flavescens*) in Chile probed in this study. Fecal samples were collected from individuals of an "urban" *O. flavescens* colony in (1) Valdivia (39° 48′ 46.84′′ S, 73° 14′ 54.212′′ W; Región de Los Ríos), and from two colonies at the Pacific coast located in (2) Punta Tiburón (40° 37′ 58.073′′ S, 73° 47′ 43.828′′ W) and (3) Caleta Cóndor (40° 46′ 23.581′′ S, 73° 51′ 7.761′′ W; Región de Los Lagos).

feces were collected and classified as individual samples (**Figure 2E**), whereas indistinct zoned and greased excrements were not included within this study. Sufficient distances were kept at any time of sampling and documentation procedures by digital drone- and GoPro®-cameras.

Coproscopical Analyses

After collection, fecal samples were stored in plastic tubes at 4°C at the Institute of Pathology of the University Austral of Chile

(UACH). Upon arrival to UACH, parts of collected feces were also preserved in 80% ethanol for detailed diagnosis of protozoan and helminth parasites. The ethanol-fixed scat samples were stored in 2 ml eppendorf tubes and according to official cooperation agreement transported to the Institute of Parasitology at the Justus-Liebig-University Giessen (JLU), Germany, for further coproscopical analyses including standard sodium acetate acetic acid formalin (SAF) technique for detection of protozoan cysts, trophozoites and oocysts, and also helminth eggs according

FIGURE 2 | "Urban" colony of South American sea lions living in the city of Valdivia, Chile. **(A)** Individuals resting at a construction site located at river Calle-Calle in immediate proximity to construction material, a truck and a portable toilette. **(B)** Dominant *Otaria flavescens* male resting at main square in front of humans and a stray dog. **(C)** Sea lion waiting for fish remains infront of the local fish market of Valdivia surrounded by cormorants and black vultures. **(D)** Three stray dogs attacking a sea lion individual at the pier. **(E)** Sampling of sea lion feces within the city of Valdivia.

to Yang and Scholten (1977). Parasite stages were determined using light microscopy (Olympus BH-52®) following diagnostic guides published before (Hermosilla et al., 2016a, 2018b). Furthermore, presence of zoonotic-relevant protozoan parasites was determined using different methods, namely carbolfuchsin fecal smears (CFS) for detection of oocysts of *Cryptosporidium* spp. (according to Heine, 1982), as well as coproantigen ELISAs (ProSpecT®, Oxoid) for detection of antigens of *Giardia* and *Cryptosporidium*. Fresh individual scat samples were also immediately used for Baermann funnel technique to detect first stage lungworm larvae (L1) of *Otostrongylus circumlitus* and *Parafilaroides* spp. according to Bowman (1995), as well as for sieving processes to isolate pre-adult and adult helminth specimens. For Baermann funnel technique, feces were grouped into clusters of five samples each. Thereby, in total 25 g of sea lion feces per cluster were swathed into two layers of gauze, put into a sieve (mesh size 100 μm) and added into a Baermann apparatus. Afterward, sample was spilled with tap water until half of gauze was covered with water. After 24 h at room temperature (RT), the clamp of the apparatus was carefully opened placing two to three drops on a microscope glass coverslip and analyzed microscopically for presence of lungworm L1. Positive cluster samples were re-examined individually. Morphology as well as morphometry of lungworm L1 was used to differentiate *O. circumlitus* from *Parafilaroides* species (Measures, 2001).

Using dissection needles, macroscopically observable nematodes and cestodes were immediately extracted out of fresh feces during field expeditions and preserved in 80% ethanol

in order to retain good morphological condition for further morphological and molecular investigation and final species identification (**Figure 3**). Sieving of feces was conducted by using metal sieves (Rems, Germany) with a 50 µm pore mesh size under continuous tap water washings.

Preparation of *Ogmogaster heptalineatus*-Eggs for Scanning Eelectron Microscopy (SEM) Analyses

Droplets isolated out of *O. heptalineatus*-SAF positive scat samples were deposited on orbital glass coverslips [10 mm in diameter (Nunc) and previously pre-coated with poly-L-lysine (Sigma-Aldrich)] according to Hermosilla et al. (2018a). Thereafter, samples were fixed in glutaraldehyde (2.5%; Merck), post-fixed in osmium tetroxide (1%; Merck), washed in distilled water, dried through CO_2 critical point procedure and sputter coated with gold particles. Prepared samples were then examined on Philips XL30® (Amsterdam, Netherlands) scanning electron microscope allocated at the Institute of Anatomy and Cell Biology (Justus Liebig University Giessen, Germany).

Morphological and Molecular Analyses of Adult Anisakid Nematodes and a Cestode

Anisakid nematodes and diphyllobothriid cestodes isolated through sieving were first photographed using a stereo microscope (Nikon SMZ25®, Tokyo, Japan). For molecular identification, proglottid of cestode and small pieces of nematode tissues were obtained from the middle part in order to preserve anterior and posterior parts of nematodes for morphological analyses and preservation as voucher specimens. Molecular identification of anisakids was conducted through PCR amplification and sequencing of a 464 bp sequence of the mitochondrial cytochrome c oxidase gene (COI) as described previously (Bowles et al., 1992).

Molecular characterization of the cestode parasite followed previously published protocols. Amplification of both the partial nuclear large subunit ribosomal RNA gene (lsrDNA) and the complete COI followed Hernández-Orts et al. (2015). Assembled contiguous lsrDNA and COI sequences were aligned with the original datasets from Waeschenbach et al. (2017) and Hernández-Orts et al. (2015), respectively. Maximum likelihood trees of individual gene datasets were estimated in IQ-TREE 1.6.8, employing the substitution models TIM3 + F + I + G4 (lsrDNA) and TIM2 + F + I + G4 (COI), chosen according to the corrected Akaike information criterion. Pairwise genetic p-distances were calculated from the total number of nucleotide differences from the lsrDNA alignment limited to *Adenocephalus pacificus* and its closely related species. Diphyllobothrium Scoticum and of the Full COI Alignment Excluding Partially Characterized Sequences.

Ethics Statement

Ethical review and approval was not required for the animal study due to the non-invasive sampling techniques applied in this study. Sample collection was conducted in accordance to the Institutional Ethic Commission and Animal Care Committee of

FIGURE 3 | Helminths isolated out of *Otaria flavescens* feces: **(A)** cestode *Adenocephalus pacificus*, **(B–D)** specimes of Anisakidae nematodes. Scale bars: **(A)** 4 cm, **(B)** 1 mm, **(C,D)** 500 µm.

the University Austral of Chile (Valdivia, Chile) and to current Chilean Animal Welfare Legislation.

RESULTS

Coproscopical Analyses

Coproscopical analyses of fecal samples of different colonies of South American sea lions in Chile detected a wide spectrum of cysts, oocysts, eggs, and lungworm L1 of endogenous parasites. In total nine different taxa, including stages of two protozoan and seven helminth parasites were found. Complete list of parasite species found in the current and previous studies is given in **Tables 1– 3**, including applied methodology and corresponding prevalences. Selected light microscopy images of parasites found in this survey are shown in **Figures 3, 4**.

Within the urban South American sea lion colony, the most prevalent gastrointestinal parasite stages found were Anisakidae gen. spp.-eggs (28.7%; **Figures 4A,B**) and Diphyllobothriidae gen. spp.-eggs (16.5%; **Figure 4C**), followed by characteristical *Ogmogaster heptalineatus*-eggs (10.4%; **Figures 4D–F**). Two further trematode taxa were identified as Trematoda indet. type 1 (5.2%; **Figure 4G**) and Trematoda indet. type 2 (9.6%; **Figure 4H**). By Baermann funnel technique, pinniped lungworm L1 of *Otostrongylus circumlitus* (14.8%; **Figure 4I**) and *Parafilaroides* spp. (7.0%; **Figure 4J**) were identified based on their morphology and size. *O. circumlitus*-L1 presented a slightly bent shape with a conically tapering end and had a mean size of 345.97 × 15.78 μm (322.01–368.57 × 14.90–16.17). Conversely, L1 of *Parafilaroides* spp. were clearly smaller, exhibited a bent posterior part of the body ending in pointed shape and reaching a mean size of 243.44 × 10.82 μm (223.14–265.04 × 10.13–11.70). In two fecal samples (1.7%), a co-infection of *O. circumlitus* and *Parafilaroides* spp. was verified.

The highest protozoan parasite prevalence was detected for *Cryptosporidium* spp. oocysts/antigens (11.3%; **Figure 4K**), followed by *Giardia* spp. cysts/antigens (5.2%; **Figure 4L**). *Giardia* and *Cryptosporidium* prevalences were confirmed via SAF-technique, CFS, and coproantigen ELISAs.

Analyses of fecal samples of *O. flavescens* collected at Pacific coast revealed the following results: The most prevalent helminth parasites detected in Pacific colony 01 were anisakids (58.2%) and Diphyllobothriidae gen. spp. (26.6%), followed by eggs of Trematoda indet. type 2 (13.1%), Trematoda indet. type 1 (5.3%), and *Parafilaroides* spp.-L1 (5.3%). Regarding protozoan parasites, most prevalent genus was *Giardia* (15.8%), followed by *Cryptosporidium* (13.2%).

Helminths detected in the Pacific colony 02 were the followings: anisakids (58.5%), Diphyllobothriidae gen. spp. (39%), and Trematoda indet. type 2 (4.9%). The most prevalent protozoan parasite in Pacific colony 02 was *Giardia* (12.2%), followed by a lower *Cryptosporidium* (7.3%) prevalence. Neither *O. circumlitus*- nor *Parafilaroides* spp.-L1 were detected in the Pacific *O. flavescens* colony 02.

In total, four out of all parasite taxa detected bear an anthropozoonotic potential: Anisakidae gen. spp., Diphyllobothriidae gen. spp., *Cryptosporidium* spp., and *Giardia* spp.

Morphological Analysis of *Ogmogaster heptalineatus*-Eggs

Light microscopy of *O. heptalineatus*-eggs allowed parasite identification to the species level according to Carvajal et al. (1983) and showed heterogeneity of filament morphology (**Figures 4D–F**). SEM analysis brought first ultrastructural insights in special egg morphology and filament windings (**Figures 5A,B**). For measurements, eggs of *O. heptalineatus* were morphologically divided in a corpus section and plait-like filaments arising from each egg pole, respectively. Corpus of eggs showed an oval stretched shape (**Figure 5A**) exhibiting a mean size of 22.72 × 11.55 μm (21.69–23.39 × 11.26–11.80), while attached elongated filaments had a mean size of 30.29 μm (27.38–34.28) each. Filaments exhibited flexible composition and no stiff structure (**Figure 4E**). In some cases, eggs with broken filaments were found (**Figure 4F**). Both filaments were equal in length and consisted of various wisps originating broad-based from each egg pole, which were frequently entangled with increasing length of filament (**Figure 5B**).

Molecular Identifications of Nematode and Cestode Species

Molecular analyses by partial sequence determination of the mitochondrial COI and BLAST of the corresponding sequences in GenBank® revealed sequences with 99% homology or more to *Pseudoterranova cattani* and to *Contracaecum ogmorhini*. In addition, sequences with a homology of 98% or more to *A. simplex* and *A. pegreffi* were found, with no higher homology to any sequences in GenBank®. Sequences belonging to *A. simplex*/*A. pegreffi* could not be further differentiated.

All species were found in the feces of urban *O. flavescens*, while only *A. simplex*/*A. pegreffi* and *P. cattani* were detected in scat samples at Pacific colonies (**Table 3**).

Analyses based on sequences amplified from the genomic DNA extracted from a cestode strobila fragment of "urban" *O. flavescens* confirmed the cestode species identity as *Adenocephalus pacificus* (**Figure 3**). The lsrDNA sequence was identical with all the other lsrDNA sequences of *A. pacificus* available (length of the alignment 1,415 bp), whereas it showed a 1.55% divergence from the closest related species of *A. pacificus* known, *Diphyllobothrium scoticum*. The phylogenetic analysis based on the COI alignment (1,563 bp; results not shown) unequivocally placed the Chilean specimen within the lineage of *A. pacificus*, sister to a specimen of *A. pacificus* from a human infection in Peru (GenBank® Accession KR269743). Pairwise uncorrected p-distances between the Chilean and the remaining specimens of *A. pacificus* in the same dataset ranged from 0.64 to 2.69%, standing in a strong contrast to the 15.4% nucleotide divergence

TABLE 2 | Prevalences (%) of protozoan and metazoan endoparasites detected in *Otaria flavescens* scat samples within this study, including comparison with previous non-invasive fecal sample monitoring studies from Chile.

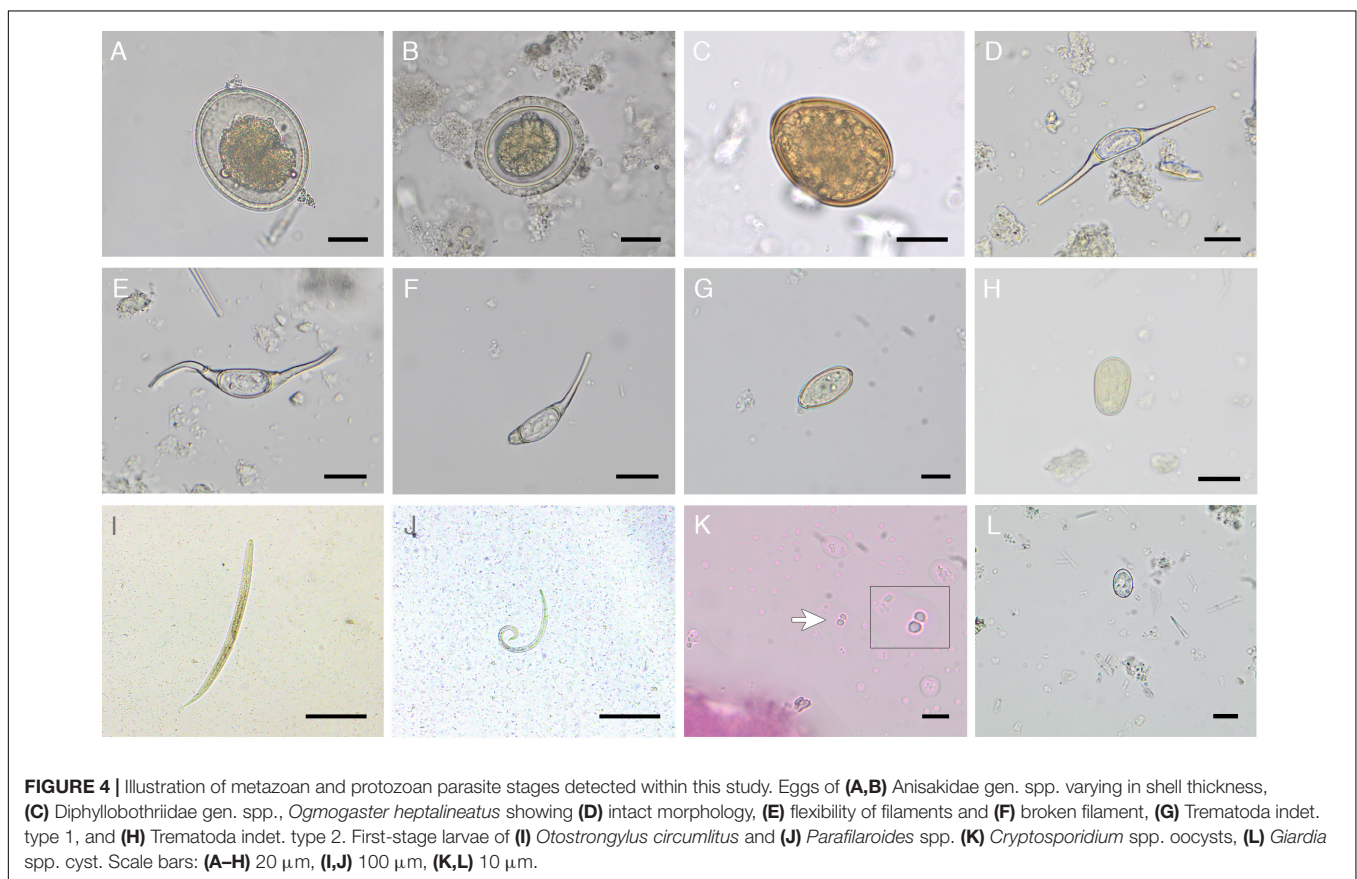|	Parasite	Stage	Technique*	Present study			Hermosilla et al. (2016a)	Hermosilla et al. (2018b)
				Urban colony	Pacific colony 01	Pacific colony 02	Urban colony	Pacific colony
				Valdivia	Osorno	Osorno	Valdivia	Comau Fjord
				n = 115	*n* = 38	*n* = 41	*n* = 40	*n* = 28
Protozoa	**Cryptosporidium spp.**	Oocysts	CFS/coproELISA	11.3	13.2	7.3	10	–
	Giardia spp.	Cysts	SAF/coproELISA	5.2	15.8	12.2	5.3	–
	Isospora spp.	Oocysts	SAF	–	–	–	5.3	–
	Neobalantidium spp.	Cysts	SAF	–	–	–	2.5	13.8
	Entamoeba spp.	Cysts	SAF	–	–	–	–	3.4
Metazoa	**Anisakidae gen. spp.**	Eggs	SAF	28.7	58.2	58.5	21[a]	34.5[a]
	Diphyllobothriidae gen. spp.	Eggs	SAF	16.5	26.6	39	13[b]	44.8[b]
	Ogmogaster heptalineatus	Eggs	SAF	10.4	–	–	–	–
	Trematoda indet. type 1	Eggs	SAF	5.2	5.3	–	2.5	–
	Trematoda indet. type 2	Eggs	SAF	9.6	13.1	4.9	–	–
	Otostrongylus circumlitus	Larvae (L1)	Baermann	14.8	–	–	–	–
	Otostrongylus sp.	Larvae (L1)	SAF	–	–	–	2.5	–
	Parafilaroides spp.	Larvae (L1)	Baermann/SAF	7.0	5.3	–	–	3.4

*Parasites detected in the current study marked in bold. *CFS, carbolfuchsin staining; SAF, standard sodium acetate acetic acid formalin-technique; Baermann, Baermann funnel technique.*
[a]*Hermosilla et al. (2016a, 2018b) identified eggs as Anisakidae gen. sp.*
[b]*Hermosilla et al. (2016a, 2018b) identified eggs as Diphyllobothriidae gen. sp.*

	Parasites	GenBank accession numbers	
		COI	lsrDNA
Urban colony	**Nematoda**		
	Anisakis simplex/A. pegreffi	–	–
	Contracaecum ogmorhini	MT941430	–
	Pseudoterranova cattani	MT941431	–
	Cestoda		
	Adenocephalus pacificus	MN967011	MN967010
Pacific colony 01	**Nematoda**		
	Anisakis simplex/A. pegreffi	–	–
	Pseudoterranova cattani	MT941432	–
Pacific colony 02	**Nematoda**		
	Anisakis simplex/A. pegreffi	–	–

FIGURE 4 | Illustration of metazoan and protozoan parasite stages detected within this study. Eggs of **(A,B)** Anisakidae gen. spp. varying in shell thickness, **(C)** Diphyllobothriidae gen. spp., *Ogmogaster heptalineatus* showing **(D)** intact morphology, **(E)** flexibility of filaments and **(F)** broken filament, **(G)** Trematoda indet. type 1, and **(H)** Trematoda indet. type 2. First-stage larvae of **(I)** *Otostrongylus circumlitus* and **(J)** *Parafilaroides* spp. **(K)** *Cryptosporidium* spp. oocysts, **(L)** *Giardia* spp. cyst. Scale bars: **(A–H)** 20 μm, **(I,J)** 100 μm, **(K,L)** 10 μm.

from the closest COI sequence of the diphyllobothriidean *Diphyllobothrium balaenopterae*.

Literature Search

Including findings of the present study, at least 50 endoparasite taxa (six protozoan and 44 metazoan taxa) associated with *O. flavescens* could be detected. Thereby, 25 taxa (one protozoan and 24 metazoan taxa) could be identified to species level, while 20 parasites (five protozoan and 15 metazoan taxa) were identified to genus level. In total, at least 25 of 50 endoparasite taxa (50%) have previously been reported to bear zoonotic potential. Taxonomy and latest classification of endoparasite names and synonyms were obtained using WoRMS Editorial Board (2020) (**Table 1**).

DISCUSSION

In the last years, development and application of non-invasive sampling techniques in the field of parasitology of marine
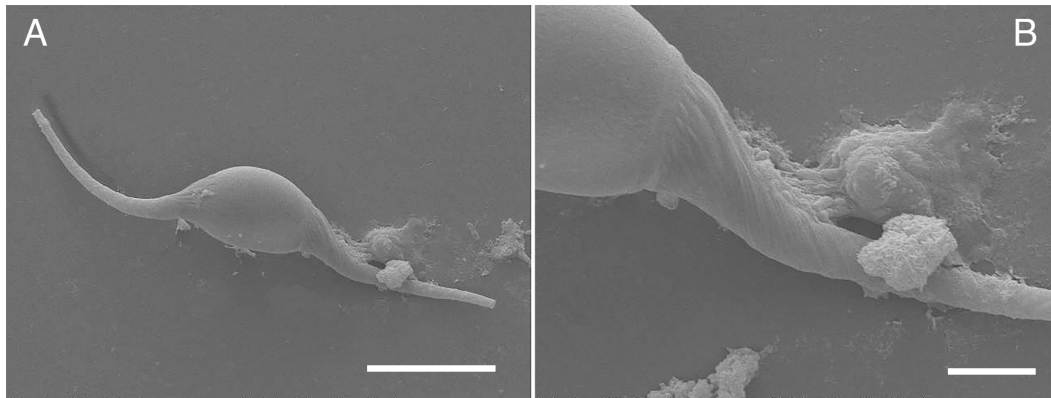
FIGURE 5 | Scanning electron microscopic images of eggs of *Ogmogaster heptalineatus*. **(A)** Overview of closed egg exhibiting filaments on both egg poles, **(B)** detailed view of emanating filament composed of various intertwined wisps. Scale bars: **(A)** 20 μm, **(B)** 5 μm.

mammals have impressively expanded the possibilities of examining the endo- and ectoparasite fauna of free-ranging animals *in vivo*, while clearly leaving the animals unmolested in their natural habitats (Kleinertz et al., 2014; Hermosilla et al., 2015; Hermosilla et al., 2016a,b, 2018a,b; de Vos et al., 2018; Ebmer et al., 2019; Vélez et al., 2019). Thereby, the present study exhibits advantages and usefulness of applied non-invasive scat collection methods, showing their applicability in the field of marine mammal parasitology.

The current study presents a detailed overview of previous publications, and simultaneously extends knowledge on the (anthropozoonotic) endoparasite fauna of South American sea lions. Coproscopical analyses revealed presence of nine different parasite taxa in individual fecal samples, including two protozoan and seven metazoan parasites, while morphological and molecular characterizations of adult helminths added the diagnosis of at least four more species, covering a wide range of parasites and corresponding well to previous reports (Hernández-Orts et al., 2013b; Hermosilla et al., 2016a, 2018b). Overall, at least 50 protozoan and metazoan endoparasite taxa have been recorded for *O. flavescens* so far (**Table 1**), while at least 25 of these taxa (50%) have to be considered as zoonotic pathogens.

Current applied coproscopical techniques (i.e., SAF, carbolfuchsin-stained fecal smears, *Giardia/Cryptosporidium* coproantigen ELISAs) reinforced proper diagnosis of the most common parasite stages (e.g., cysts, oocysts, and eggs) in marine mammal scat samples (Kleinertz et al., 2014; Hermosilla et al., 2015, 2016a,b, 2018a,b; Vélez et al., 2018, 2019). Furthermore, sieving- and Baermann funnel-methods of freshly shed feces permitted detection of spontaneously excreted adult ascarids and cestodes (scolex and strobila) as well as detection of *O. circumlitus/Parafilaroides* spp.-L1. During sample-taking process, feces were carefully searched for macroscopically visible adult helminths, which were immediately extracted and preserved in 80% ethanol to protect morphological features. Together with sieving of feces in the laboratory, this sample-taking technique constitutes a crucial possibility to obtain

spontaneously excreted adult helminths in good morphological condition in the field of non-invasive wildlife parasitology for a broad spectrum of possible analyses (Hermosilla et al., 2018b).

Overall, the highest prevalences found in the synanthropic South American sea lion colony were recorded for eggs belonging to the helminth families Anisakidae (28.7%) and Diphyllobothriidae (16.5%), which is in good accordance to previously reported prevalences (21 and 13%, respectively) detected within the same population (Hermosilla et al., 2016a). In contrast, *O. flavescens* from Pacific coast colonies showed much higher prevalences for these two helminth groups [anisakids (58.2/58.5%) and diphyllobothriids (26.6/39%)]. These high helminth prevalences are in accordance to high prevalences detected in scat samples of *O. flavescens* collected in the Comau Fjord in Northern Patagonia, Chile (Hermosilla et al., 2018b). In this former study a prevalence of 34.5 and 44.8% were found for anisakid nematodes and diphyllobothriid cestodes. Interestingly, urban South American sea lions differ from their conspecifics in their food spectrum, and this could explain diverging parasite prevalences. Since natural foraging behavior of urban sea lions is less manifested than in animals from Pacific coastal areas, feedings of sea lions by marketeers and eating mainly fish remains and human waste at Valdivia's fish market constitute basic food resources for members of this colony. Fish remains are mainly composed of heads and dorsal and caudal fins of various species of marine fish, which are not reported as manifestation sites of plerocercoids of Diphyllobothriidae or infective third larvae of Anisakidae within intermediate or paratenic hosts (Dezfuli et al., 2007; Moravec, 2009; Hernández-Orts et al., 2013a). Consistently, anisakid larvae have been reported to occur in different marine fish tissues sold at markets in Valdivia (Torres et al., 2000).

Irrespective of prevalence levels, both helminth parasites exhibit considerable anthropozoonotic potential worldwide, and should be considered as neglected zoonoses (Chai et al., 2005). All anisakid species found in this study (*A. simplex/A. pegreffi*, *C. ogmorhini*, and *P. cattani*) represent etiological agents of human anisakiasis (Acha and Szyfres, 2003). Humans constitute accidental hosts for these parasites ingesting infectious

third-stage larvae (L3) via raw or insufficient cooked fishes or cephalopods (Chai et al., 2005). The *A. simplex* sensu lato (s.l.) complex is composed of three species, namely *A. simplex* sensu stricto (s.s.), *A. pegreffi*, and *A. berlandi* (Abattouy et al., 2016; Llorens et al., 2018). Since hybridization needs to be considered in areas of sympatry, differentiation between *A. simplex* s.s. and *A. pegreffi* is difficult (Abattouy et al., 2016). Focusing on molecular characterization based on mitochondrial COI, differentiation between *A. simplex* s.s. and *A. pegreffi* was not possible within the current study. The complex *P. decipiens* constitutes a species group composed of at least six species differing in their geographic localization (Hernández-Orts et al., 2013a). *P. cattani* solely occurs in the Southern Hemisphere and was recently reported as emerging infection in Chile causing oropharyngeal pseudoterranovosis in humans (Torres et al., 2007; Weitzel et al., 2015). The majority of these cases was reported to be related to consumption of raw fish dishes, e.g., ceviche, which is in accordance to other regions of South America (Menghi et al., 2020). Anisakid larvae belonging to the genus *Pseudoterranova* were also found in ceviche dishes sold at Valdivian fish market (Torres-Frenzel and Torres, 2014).

Diphyllobothriosis constitutes one of the most neglected fish-borne zoonosis worldwide and is caused by adult species of the genus *Dibothriocephalus*, *Diphyllobothrium*, and *Adenocephalus* parasitizing mainly piscivorous birds and mammal host species (Chai et al., 2005; Scholz et al., 2009; Kuchta et al., 2015). Infectious stage for final hosts (including humans), the plerocercoid, is located in different tissues of anadromous, freshwater and marine fishes, which are representing the second-intermediate- or paratenic hosts (Chai et al., 2005; Scholz et al., 2009). Thereby, *Adenocephalus pacificus* (syn. *Diphyllobothrium pacificum*) constitutes the most abundant anthropozoonotic cestode species occurring in South America (Kuchta et al., 2015) and was reported for *Otaria flavescens* in Chile (Cattan et al., 1977) and Peru (Mondragón-Martínez, 2017; Naupay et al., 2019), where most human cases occur (Kuchta et al., 2015). Several human infections were also described for Chile (Mercado et al., 2010).

Anthropozoonotic protozoan parasites of the genus *Giardia* and *Cryptosporidium* were already reported during the first parasitological monitoring in Valdivias "urban" colony (Hermosilla et al., 2016a). While both water-borne parasites were already described for other free-living sea lion species, e.g., the Californian sea lion (*Zalophus californianus*) (Deng et al., 2000) and the Australian sea lion (*Neophoca cinerea*) (Delport et al., 2014), the results of the current study showed the presence of *Giardia* and *Cryptosporidium* in two free-ranging colonies of South American sea lions apart from human settlement for first time. Interestingly, *Giardia* and *Cryptosporidium* were found at a higher prevalence in free-ranging colonies than in the urban colony showing that these anthropozoonotic protozoan parasites already circulate in sea lion colonies located at the Chilean pacific coast. Up to date, the "urban" sea lion colony is still staying in interchange with other *O. flavescens* colonies at the Pacific coast, and younger males could return to breeding colonies. Beside contamination of river Calle-Calle with environmental resistant *Giardia*-cysts and *Cryptosporidium*-oocysts, it is conceivable

that these parasite stages might be transported out of the city by regular annual migration during mating season. More importantly, human studies confirmed presence of *G. intestinalis* not only in different age classes of patients in Valdivia but also in school children living in coastal localities nearby Valdivia (Torres et al., 1992; Navarrete and Torres, 1994) associated with inadequate sanitary installations. South American sea lions most probably become infected from cysts and/or oocysts derived from sewage waters or by livestock or vice versa. Consistently, in the city of Valdivia, direct and indirect interspecific contact between humans, sea lions and a vast amount of domestic and wildlife animal species constitutes a crucial factor for transmission of parasite stages and maintenance of zoonotic parasite life cycles within the One Health concept.

Additionally, pinniped-specific parasites were also found such as the intestinal trematode *Ogmogaster heptalineatus*. *O. heptalineatus* belongs to the family Notocotylidae and was firstly described in 1983 from South American sea lions in Chile (Carvajal et al., 1983). Considering the fact that Sepúlveda et al. (2015) already showed the presence of *O. heptalineatus* within the urban sea lion colony by obtaining adult specimens during dissection of a sea lion carcass, *Ogmogaster* eggs detected within this study were determined on species level using morphological description (Carvajal et al., 1983). Eggs of *O. heptalineatus* are characterized by plait-like filaments, which arise from each egg pole and are distinctly shorter when compared with eggs of other marine *Ogmogaster* species, e.g., *O. trilineatus* (Rausch and Rice, 1970). Regarding the role of similar appendices manifested by the notocotylid species *Notocotylus attentuatus*, it was hypothesized that these filaments might be essential for forming egg clusters and be more resistant to cecal peristalsis than single eggs (Graczyk and Shiff, 1993). Moreover, Dönges (1962) supposed that filaments of *N. ralli* constitute a helpful tool for floating in different depths of water thereby exhibiting a higher chance of ingestion by adequate mollusk intermediate hosts, whereas life cycle is not completely explored yet. Interestingly, *O. heptalineatus* eggs were solely present in feces of "urban" sea lions, although members of genus *Ogmogaster* have so far been reported to exclusively infect whales and pinnipeds living in marine habitats.

Up to date, trematodes of the genera *Ascocotyle* (Morgades et al., 2006; Hernández-Orts et al., 2012; Pereira, 2012; Pereira et al., 2013), *Lobatostoma* (possibly fish parasite detected after gastrointestinal passage; Calderón-Mayo, 2015), *Ogmogaster* (George-Nascimento and Carvajal, 1981; Carvajal et al., 1983; Sepúlveda et al., 2015), *Stephanoprora* (Morgades et al., 2006; Pereira et al., 2013), and *Zalophotrema* (only one report in captivity; Petrov and Chertkova, 1963) have been reported for South American sea lions (**Table 1**). In the current study, eggs of two different trematode taxa were specified on clade level determined as Trematoda indet. types 1 and 2. Eggs of Trematoda indet. type 1 were already detected by Hermosilla et al. (2016a) in synanthropic sea lion feces, and size and shape are morphologically similar to eggs of members of trematode families Opisthorchiidae and Heterophyidae (Ditrich et al., 1992), which includes the genus *Ascocotyle*. Members of Opistorchiidae have never been reported parasitizing *O. flavescens* so far, however,

other pinnipeds were described to function as definitive hosts (Chai et al., 2005; Heckmann and Halajian, 2012; Neimanis et al., 2016). Depending on species and localization of adult trematodes, dimension of infections in marine mammals can range from low enteritis up to necrosis of parenchyma of affected organs (Geraci and St. Aubin, 1987).

Baermann funnel technique, a gold standard method for detection of first-stage lungworm larvae revealed two different parasites: *Otostrongylus circumlitus* and *Parafilaroides* spp. Both lungworm taxa were identified using morphological descriptions (Measures, 2001) and were detected in the synanthropic sea lion colony, whereas only *Parafilaroides* spp. L1 were found in Pacific sea lion colony 01. Data on lungworm infections in sea lions (subfamily Otariinae) are still scarce and exclusively caused by *O. circumlitus* (Kelly et al., 2005) and members of the genus *Parafilaroides* (Dougherty and Herman, 1947; Sweeney and Gilmartin, 1974; Greig et al., 2005), which have both been documented for South American sea lions before (Gonzales-Viera et al., 2011; Hermosilla et al., 2016a, 2018b). *O. circumlitus* (Metastrongyloidea: Crenosomatidae) constitutes the only described species within the genus *Otostrongylus* (Leidenberger and Boström, 2009) and has been reported to mainly parasitize principal airways causing distinct clinical signs especially in juvenile phocid seals and fatal cases have also been published (Gulland et al., 1997). In this study, first stage larvae of *O. circumlitus* reached a mean size of 345.97 × 15.78 μm and were thereby longer than maximum length published for genus *Parafilaroides* (304 μm; Measures, 2001). Measurements laid below references of *O. circumlitus* first-stage larvae, whereas length of larvae can vary according to host species (Bergeron et al., 1997). Consistently, the host immune response, nutritional status, age and body size can influence final adult parasite length and width *in vivo* (Morand et al., 1996). For the California sea lion, a case report of verminous pneumonia and bronchitis caused by *P. decorus* was published (Fleischman and Squire, 1970). Manifesting a curved and tapering posterior end, first stage larvae (L1) of *Parafilaroides* spp. detected within the current study exhibit a similar morphology to lungworm larvae found in Galapagós sea lions (*Zalophus wollebaeki*), which were identified as *Parafilaroides*-like nematodes (Walden et al., 2018). Measurement of our *Parafilaroides* spp. larvae were in good accordance to published morphometric data (Measures, 2001). As already reported by Hermosilla et al. (2016a), many synanthropic male sea lions regularly showed periods of extensive coughing and expectoration and extend rhinorrhea also during the current study indicating a possible clinical manifested otostrongylosis and/or parafilaroidosis. Sampling of expelled mucus or nasal discharge are planned for future monitoring surveys displaying a suitable collection technique to reveal either pre-adult/adult- or L1-stages of lungworms *in vivo* in a non-invasive and unmolested way.

The current study delivers new data on the endogenous parasite fauna of different colonies of free-living South American sea lions and represents the continuation of a monitoring program on anthropozoonotic parasites within One Health-/Global Health-concepts. Survey revealed different parasite taxa, including parasites bearing anthropozoonotic potential, thus evidencing importance of management measures avoiding direct contact with feces or products containing infectious parasite stages as well as regular monitoring programs to avoid spillovers and human infections. By detecting a broad spectrum of parasite stages (cysts, oocysts, eggs, L1 as well as adult helminths), the current study clearly illustrates a successful application and functionality of non-invasive methods in the field of parasitology research of marine mammals.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study due to the non-invasive sampling techniques applied in this study. Sample collection was conducted in accordance to the Institutional Ethic Commission and Animal Care Committee of the University Austral of Chile (Valdivia, Chile) and to current Chilean Animal Welfare Legislation.

AUTHOR CONTRIBUTIONS

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