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Parasitic load of the Pacific mackerel, *Scomber japonicus* (Pisces: Scombridae) from Northwestern Baja California, Mexico

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Summary

Globally, the exploitation of small pelagic fish, like Pacific mackerel is of great importance due to food industry demand. However, there are few studies regarding its parasites load and there are no in this geographic zone. This study aimed to assess the parasitic composition, some temporal changes (during spring and summer) in abundance, prevalence and intensity of infection parasitic of the Pacific mackerel (*Scomber japonicus*) from Todos Santos Bay, Baja California, Mexico. The parasite fauna of the Pacific mackerel consisted of 1930 parasites (1413 in spring and 517 in summer) distributed in the follow taxa: an Tetraphyllidea (Cestoda), *Kuhnia scombri* (Monogenea), Didymozoidae (Digenea), *Anisakis* sp. (Nematoda), *Rhadinorhynchus* sp. (Acanthocephala) and *Caligus pelamydis* (Copepoda). The nematodes parasite were the most abundant both in spring with a mean abundance of 27.6 parasites and in summer 8.2 parasites compared with the other taxa like Cestoda, Monogenea, Digenea, Acanthocephala and Copepoda (P = 0.003). The mean intensity of the nematodes in spring and summer was 28.1 and 13.4, respectively. The nematodes prevalence was 90 % in spring and 60 % in summer. In general, the parasite load is more abundant in spring than summer. In summer, absence of taxa as Cestoda and Copepoda were registered. Nematode larvae were present in the fish guts mesentery and inside of the stomach, pyloric caeca, intestine. Also the nematodes were found in the liver, muscle and gonads. The most affected organ by nematodes was the intestine mesentery. The most predominant parasite of this study has been *Anisakis* sp. during spring.

Keywords: Pacific mackerel; helminth parasites; *Anisakis*

Introduction

The Pacific mackerel (*Scomber japonicus*) is a coastal pelagic species in the north-eastern Pacific that ranges from south-eastern Alaska to Banderas Bay, México, including the Gulf of California (Hart, 1973). They are frequent from Monterey Bay (California, USA) to Cabo San Lucas (Baja California Sur, México). Pacific mackerel usually migrate from south to north in summer and overturn the migration in winter and tend to move from inshore during the spawning season, March to May (30 km), to offshore as far as 400 km (Frey, 1971; MBC, 1987; Allen *et al*., 1990; Lo *et al*., 2010). Pacific mackerel in recent times have been popular in Baja California, México and this is demonstrated by the historical series of fishing production where an increase of 8 tons to 12,487 tons annual catch between 2009 and 2018 was observed (CONAPESCA, 2020).

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The Pacific mackerel is fished commercially by purse-seine vessels (Crone *et al*., 2009). Mackerels as others small pelagic fish have a significant role in marine ecosystems as a primary consumer and represent an intermediate host for many parasites. Also can be prey in the food chain, which allows the transmission of parasites to the definitive hosts (Baldwin *et al*., 2012; Del Rio-Zaragoza *et al*., 2018).

Nevertheless, few parasites studies in small pelagic fishes from Baja California are available and focused in the Pacific sardine, *Sardinops sagax* (Baldwin *et al*., 2011, 2012; Sánchez-Serrano & Cáceres-Martínez, 2017; Del Rio-Zaragoza *et al*., 2018). This lack of parasitological studies in others small pelagic fishes are noteworthy. Information on parasitic fauna of the Pacific mackerel in this geographic region is limited. Therefore, research on this direction will be helpful to characterize the parasitic load of this important commercial species. Consequently this study aimed to assess the parasitic composition, temporal changes (spring and summer) in abundance, prevalence and intensity parasitic in the Pacific mackerel (*Scomber japonicus*) from Todos Santos Bay, Baja California, Mexico.

Materials and Methods

Wild Pacific mackerel (n =107) were collected during spring (n = 47, April 2017) and summer (n = 60, August 2017) in Todos Santos Bay (31°40' to 31°56'N, 116°36' to 116°50'W, Fig. 1).

At this time, the seawater temperature ranges from 16 to 18°C, before the highest temperatures were registered in early September (19 to 23°C; Del Rio-Zaragoza *et al*., 2021). The Bay is affected by the California Current System. This current transports less saline and cool waters with high dissolved oxygen levels from the Polar Regions in direction to the equator. The current speed is typically less than 25 cm s^{-1}. During spring and summer, it flows towards the equator off California and the northern Baja California Peninsula (Mateos & Marinone, 2017).

All mackerels were transported to the laboratory in our facilities at the Universidad Autonoma de Baja California (UABC), in plastic bags (10 – 15 fish per bag) with 50 L of seawater at 19.2 ± 1.4°C, and oxygen supply >7 mg L^{-1} (Del Rio-Zaragoza *et al*., 2018). Fish collected were released in three 10m^3 tanks (during spring 15 fish by tank and during summer 19 fish by tank) with open flow and total water change twice per day. The next day after arriving at the laboratory, a commercial diet (crude protein 46 %, crude fat 16 %, 3 mm granule size,) was offered *ad libitum* (Three times a day). During this time tanks were supplemented with constant aeration and maintained at ambient temperature.

Every day, leftover food and faeces were removed from the tanks. The average water temperature in the tank was 20 ± 1.25 °C, dissolved oxygen was 7.45 ± 0.51 mg L^{-1} and salinity 33 ± 0.3 PSU. In each captured time fish were maintained as was mentioned above and then sampled in a period of 10 days for parasitic analysis.

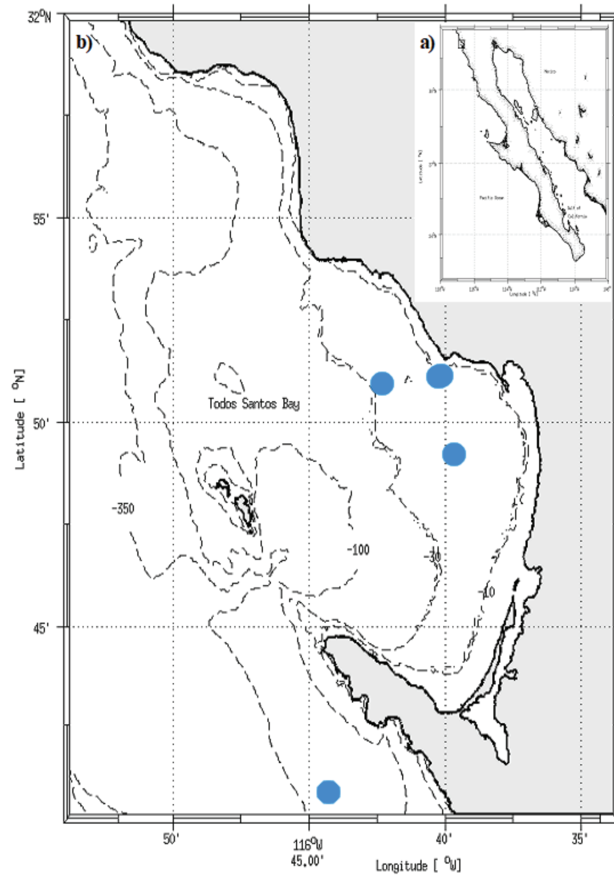

Fig. 1. Study area. a) Baja California Peninsula, Mexico, and b) detailed view of the Todos Santos Bay and bathymetry. The marks in blue represent the sampling points.

All the fish were examined under the stereomicroscope (Zeiss, Stemi 2000-C) for ectoparasites on the skin, mouth and gills. Endoparasite extraction was carried out by dissecting viscera. Each organ, i.e. the gut, pyloric caeca, intestine, gonads, liver and heart, was checked for parasites, under the stereomicroscope (10.5X). The extraction and processing followed Pérez-Ponce de León *et al*. (1999). The parasites found were determined taxonomically to the lowest possible level for Cestoda (Avdeeva & Avdeev, 1989), Monogenea, (Yamaguti, 1968), Digenea, (Montgomery, 1957), Nematoda, (Smith 1983, Anderson, 2000) Acanthocephala (Arai, 1989), and Copepoda (Cressey & Cressey, 1980; Kabata, 2003). Condition factor (CF) was calculated as follow: CF= 100 × body weight (g) body length$(cm^3)^{-1}$. Prevalence (P), mean abundance (MA), and mean intensity (MI) of infection were calculated according to Bush *et al*. (1997).

The results are presented as the means ± standard deviation (SD). All the data were first tested to confirm a normal distribution and homogeneity of variance. As the data do not show a normal distribution, rank sum tests were used to analyze differences in parasite fauna and biological indices between spring and summer. To determine whether the parasitic load is influenced by the host

Table 1. Biological index of Pacific mackerel (*Scomber japonicus*) sampled during spring and summer. Values represent means ± standard deviation; and *P* values from rank sum tests are also provided. Means with different superscripts letters are significantly different (*P* < 0.05) within the same parameter. CF=condition factor.

Parameter	Spring (n=47)	Summer (n=57)	*P* Value
Mean weight (g)	117.42 ± 42.33	118.12 ± 63.16	0.487
Mean length (cm)	24.49 ± 2.06	22.75 ± 4.69	0.071
CF	0.769 ± 0.10	0.936 ± 0.289	<0.001

size Spearman rank correlation analyses were performed. All statistical analyses were performed using Sigma Stat 4.0 software (Systat Software, Inc., San Jose, CA, USA). The results were considered significant at *P* < 0.05.

Ethical Approval and/or Informed Consent

All procedures in the present study were conducted and authorized according to the UABC animal ethics committee (protocol UABC-IIO 00034/21).

Results

A total of 107 mackerels were collected in Todos Santos bay. The data about weight and length of the fish sampled in spring and summer are given in Table 1 and no statistical differences (*P* > 0.05) were found. The condition factor of mackerels was compared, and the values were different (*P* < 0.05) in spring and summer. Fish during summer show a higher factor condition compared with the other fish groups in spring (Table 1). Whereby 79.81 % of the specimens presented at least one parasite taxon. Correlation between fish length and the parasitic load was $r_s = 0.265$, ($p < 0.05$).
In the parasitological analysis, 1930 parasites (1413 in spring and 517 in summer) in total were collected and distributed in the following taxa: Tetraphyllidea (Cestoda), *Kuhnia scombri* (Monogenea), Didymozoidae (Digenea), *Anisakis* sp. (Nematoda), *Rhadinorhynchus* sp. (Acanthocephala) and *Caligus pelamydis* (Copepoda). This represents 0.05, 1.5, 4.1, 93.8, 0.1 and 0.3 % respectively of the overall parasites found in the Pacific mackerel (Table 2).
In general, the parasitic load found is higher in spring than in summer. In the summer, the absence of taxa as Cestoda and Copepoda were registered (Table 3). From all the parasites found, the anisakid nematodes were the most abundant throughout the study (Table 3). However, in the summer there is a statistically significant decrease (P = 0.003) in the anisakid nematodes abundance and intensity. The mean intensity of the nematodes in spring and summer was 28.2 and 13.9, respectively. The nematodes prevalence was 90.1 % in spring and 60.2 % in summer (Table 3).
Below is the information on the parasite species found in *S. japonicus.* It provides the morphological traits that characterize and differentiate them from other congeneric species.

Cestoda

Tetraphyllidea Carus, 1863

Cestode was identified to this level because it was recorded only as larvae in the intestine of the mackerels collected from Todos Santos (Table 2). It was incorporated into the order Tetraphyllidea

Table 2. Parasite species of the Pacific mackerel (*Scomber japonicus*) during Spring and Summer from Todos Santos bay, Baja California, Mexico. PN = parasite number, DH = distribution in the host, G = gills, S = stomach, IC = intestinal caeca, I = intestine, L = liver, MU = muscle, GN = gonads, M = mesentery, ; * = ectoparasite; ** = endoparasite

	Spring		Summer	
Parasite species	PN	DH	PN	DH
Monogenea				
Kuhnia scombri *	28	G	1	G
Digenea				
Didymozoidae**	80	S, IC, I	0	---
Cestoda				
Tetraphyllidea**	1	I	0	---
Nematoda				
Anisakis sp.**	1297	M, S, IC, I, L, MU, GN	515	M, S, IC, I, L
Acanthocephala				
Rhadinorhynchus sp. **	1	I	1	I
Copepoda				
*Caligus pelamydis**	6	G	0	---

Table 3. Prevalence (P%), Abundance (A), and Intensity (I) of the parasites found in the Pacific mackerel *Scomber japonicus* collected from Todos Santos" bay, Baja California, during Spring and Summer

	Spring (n=35)			Summer (n =60)		
Parasite species	P %	A	I	P %	A	I
Monogenea						
Kuhnia scombri *	4.9 ± 16.6	0.59 ± 1.75	2.8 ± 2.9	0.05 ± 0.41	0.01 ± 0.12	1 ± 0
Digenea						
Didymozoidae**	4.21 ± 14.2	1.7 ± 6.21	10 ± 12.6	1.3 ± 3.13	0.33 ± 0.7	1.54 ± 0
Cestoda						
Tetraphyllidea**	0.11 ± 0.81	0.02 ± 0.14	1 ± 0	---	---	---
Nematoda						
Anisakis sp.**	90.1 ± 21.9	27.6 ± 29.9	28.1 ± 30	60.2 ± 48	8.25 ± 15	13.4 ± 16.5
Acanthocephala						
Rhadinorhynchus sp.**	0.07 ± 0.52	0.02 ± 0.14	1 ± 0	1.66 ± 12.9	0.01 ± 0.1	1 ± 0
Copepoda						
*Caligus pelamydis**	0.62 ± 2.9	0.12 ± 0.53	2 ± 1	---	---	---

because of the morphology and disposition of the four sessile bothria that form the scolex of these larvae (Avdeeva & Avdeev, 1989).

Digenea

Family Didymozoidae Monticelli, 1888

Didymozoidae is the most difficult group of digeneans to identify because of the morphology. Since, species, genera and subfamilies differ only by very insignificant characters. For the morphology description of metacercariae presence or absence of acetabulum sucker, a pharynx, a stomach, and glandular cells in the region of bifurcation, the shape of the intestine branches and chambers are the principal characteristics of interest (Nikolaeva, 1981). The larval forms of the family Didymozoidae in this study were occasionally found in the fish stomach, gut and intestinal caeca (Table 2). In order to achieve the finest possible level of identification, generally the DNA sequences are generated. But, in this study such kind of analysis was not possible.

Monogenea

Mazocraeidae Price, 1936

Monogenea were found in the fish gills (Table 2). Specimens found were identified as *Kuhnia scombri* (Kuhn, 1829), based on the characteristics follow: the prohaptor has two elliptical, septate,

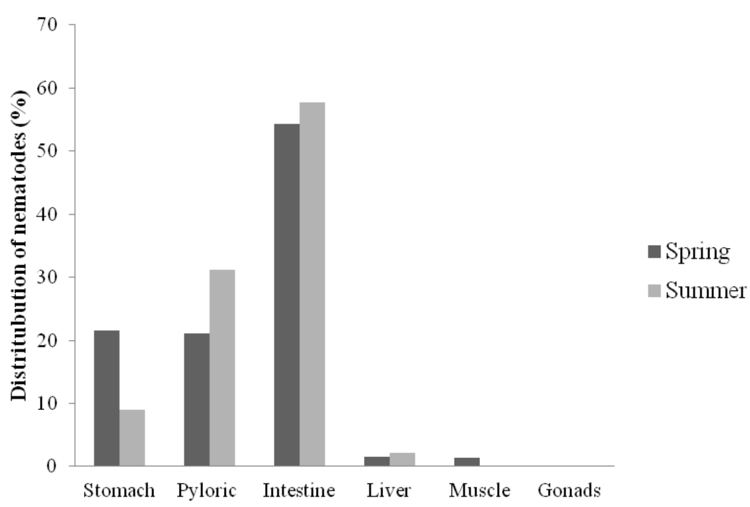

Fig. 2. Distribution of nematodes in different organs of the Pacific mackerel (*Scomber japonicus*) during Spring and Summer from Todos Santos bay, Baja California, Mexico.

intrabuccal suckers; below the suckers is a small pharynx, a relatively long oesophagus and two inconspicuous intestinal caecums, usually covered by the vitellogenic glands. The genital atrium is round and is characterised by large, recurved hooks on both sides and other hooks of the same shape but smaller inwards. The testes are numerous and the vermiform ovary is located pretesticularly. The uterus has spindle-shaped eggs, which have a filament at each pole. There is no vagina. The opisthaptor is short, rounded and has four pairs of symmetrically positioned claws, the first three of which are symmetrically positioned. At the end of the opisthaptor there are two pairs of macro-hooks with a fine, recurved tip, directed forward. Between these hooks are two microhooks. (Yamaguti,1968)

Nematoda

Anisakidae Hartwich, 1974

Anisakinae Railliet and Henry, 1912

Larvae of the anisakid nematodes were found in the fish guts mesentery and inside of the stomach, pyloric caeca, intestine. Also the nematodes were found in the liver, muscle and gonads. The most affected organ by nematodes was the intestine mesentery (Fig. 2) and they were assigned to the most common genera of this family, based on the characteristics of the digestive tract, position of the excretory pore opening and structures of the cephalic region. This genera was *Anisakis* Dujardin, 1845. The main characteristic of these larvae is the presence of a very elongated ventricle and the lack of intestinal caecae. No have interlabia and between the ventrolateral lips is the chitinous tooth situated anteriorly, having the excretory pore opening at the level of the nerve ring. Rectal canal short, oblique to the anus and surrounded by three rectal glands. Conical tail with mucron (Fig 3). The reported characteristics have been reported by the following Anderson (2000) and Sanches-Serrano & Cazares-Martinez (2017). The most predominant parasite of this study has been *Anisakis* sp. during spring (Table 2).

Fig. 3. *Anisakis* sp. found in the Pacific mackerel (*Scomber japonicus*). The anterior end with the boring tooth (a), ventriculus (b), and Posterior end with anus (c).

Fig. 4. *Rhadinorhynchus* sp. found in the Pacific mackerel (*Scomber japonicus*).

Acanthocephala

Rhadinorhynchidae Travassos, 1923

The specimens found were incorporated into the genera *Rhadinorhynchus* Luhe, 1911 (Fig.4); because of the morphology that presented these genera such as trunk elongate, subcylindrical, somewhat enlarged anteriorly, with one or two fields of body spines separated by an spinose region; body spination generally covering a triangular surface with lower point ventral; hypodermis with numerous fragments of giant nuclei; main lacunar canals lateral, united by anastomoses forming a network; proboscis very long, claviform, armed with 8-26 longitudinal rows of 8-37 hooks each; hooks showing distinct dorsoventral asymmetry, with ventral hooks stouter, larger; proboscis receptacle long, double-walled, with ganglion at mid-level; lemnisci digitiform, always distinctly longer than receptacle; male genitalia occupying posterior half of trunk; testes two, ovoid to elongate, tandem, contiguous; cement glands 2-8, club-shaped; gonopore terminal in both sexes; eggs with large polar prolongations of middle membrane (Arai, 1989). The genera *Rhadinorhynchus* of *S. japonicus* were found inside of the intestine (Table 2).

Copepoda

Caligidae Burmeister, 1835

Only females of *Caligus pelamydis* Kroyer, 1863 were collected from the gills of the *S. japonicus* (Fig 5), and can be separated from other species of *Caligus* found on scombrids by the following combination of characters: cephalon 40 – 45 percent of total length; abdomen 23 – 25 percent of total length; second and third segments of leg 2 endopod each with a large patch of fine spinules along outer edge; leg 4, 3-segmented with last segment produced distally to give segment a triangular shape and prominent fringes at bases of all setae, times of sternal furca spatulate and as wide or wide than base (Cressey & Cressey, 1980; Kabata, 2003)

Discussion

In this study we assessed the parasitic composition, temporal changes (spring and summer) in abundance, prevalence and intensity parasitic in the Pacific mackerel (*S. japonicus*) from Todos Santos Bay, Baja California, Mexico. In the present study, the fish condition factor during spring shows a low value that increases into the summer. Probably, as a result that in Todos Santos Bay during spring, the surface water is rich in nutrients and chlorophyll, while during September and October, there is an increase in the flow of warmer and surface water that is poor in nutrients concentrations and chlorophyll by the decrease in coastal upwelling (Espinosa-Carreón *et al*. 2001). This could be the result that the parasite found (Cestoda: Tetraphyllidea; Monogenea: *Kuhnia scombri*; Digenea: Didymozoidae; Nematoda: *Anisakis* sp; Acanthocephala: *Rhadinorhynchus* sp. and Copepoda: *Caligus pelamydis*) was more abundant in spring than summer.
In summer, the absence of taxa as Cestoda and Copepoda were

Fig. 5. Female of *Caligus pelamydis* found in the Pacific mackerel (*Scomber japonicus*).

registered. Consequently, some patterns of the parasitic fauna were observed, as follows: a 100 % prevalence during spring and a 63 % prevalence during summer both with a high number of nematodes larval stages. The most predominant species has been *Anisakis* sp., while in other latitudes patterns of the parasitic fauna in the Pacific mackerel are similar at the present work with some variations in the parasites species composition and also in terms of prevalence and abundance.

Published information about metazoan parasites of the Pacific mackerel have been carried out in Argentina where nine parasites species (*Kuhnia scombri*, *Opechona* sp., *Nematobothrium* sp., *Hysterothylacium* sp., *Anisakis* sp., *Contracaecum* sp., *Pseudoterranova* sp., *S. pleuronectis* and *Corynosoma australe*) were recorded (Cremonte & Sardella, 1997). Thus, Alves *et al*. (2003) reported fifteen species of parasites. Digeneans and the nematodes were the majority of the parasite specimens collected in mackerel from the coastal area of the state of Rio de Janeiro (Brazil). Subsequently, Oliva *et al*. (2008) found for the area of Rio de Janeiro (Brazil, 15 species of parasites, previously reported by Alves *et al*. (2003), Antofagasta (Chile, 14 species of parasites) and Callao (Peru, 11 species of parasites), the dominance of endoparasites, mainly digeneans. Other study realized by Cruces *et al*. (2014) in Perú reported 12 parasite species (*Clavellisa scombri, Ceratothoa gaudichaudii*, *Prodistomum orientalis*, *Koellikeria* sp., *Maccallumtrema* sp., Didimozoidea gen sp., *Ovarionematobothrium saba*, *Nematobothrium scombri*, *Scolex pleuronectis*, *Anisakis simplex*, *Contracaecum* sp. and *Rhadinorhynchus pristis*).

In our study, we found that some parasites were similar to the reported in the Pacific mackerel populations from the South Pacific Moreover, it is well known that populations from the South Pacific and North Pacific coast of America do not overlap (Stepian & Rosenblatt, 1996). Oliva *et al*. (2008) appoint that only two parasites specific to *Scomber* (the monogenean *K. scombri* and the copepod *C. scombri*) are present in all the localities from the Atlantic and Pacific Ocean and offers one of the rare instances among parasites of discontinuous distribution. Whereas the copepod *C. pelamidys* have a distribution along the Pacific coast of America (Love & Moser, 1983; Oliva *et al*., 2008). On the other hand, we have not found parasite species commonly found in the South Pacific such as *Opechona* spp. (Digenea) that was present with high prevalence and abundance (Cremonte & Sardella, 1997). At difference of the reported in the Pacific mackerel collected in Todos Santos Bay, other species of Cestoda, Monogenea, Digenea, Nematoda, Acanthocephala and Copepoda recorded by Love and Moser (1983) were absent because they correspond to parasites of *S. japonicus* recorded in other geographical regions outside this study area, e.g. fish collected in South Atlantic, Japan, Europe, etc. However, the results in our study are consistent with others fish species in this same geographic region where the highest prevalence (>75 %) for the nematodes (anisakids) were reported in the Baja California rockfish (*Sebastes auriculatus*, *S. chlorostictus*, *S. umbrosus*, *S. miniatus*, *S. atrovirens*, *S. constellatus*, *S. serranoides*, and *Scorpaena guttata*), but with an abundance and an intensity <6 parasites (Rodríguez-Santiago *et al*., 2014, 2016, 2020). Also the California halibut (*Paralichthys californicus*) presented high prevalence and an abundance <6 parasites (Castillo-Sanchez *et al*., 1998). The abundance and intensity date of nematodes mentioned above were lower compared to reported in this study with the Pacific mackerel. In the case of the Pacific sardine (*Sardinops sagax caeruleus*) from Todos Santos bay, the prevalence (>90 %)

of the parasites is dominated by trematodes (*Miosaccium ecaude*, *Parahemiurus merus*, and *Bucephalus* sp.) and nematodes (*Anisakis* sp. and *Hysterothylacium* sp.) and was higher in the winter-spring season (Sánchez-Serrano & Cáceres-Martínez, 2017; Del Rio-Zaragoza *et al*., 2018). It has been attributed to the high prevalence of the third larval stage of anisakids in fish of an ichthyophagous diet (Ferre, 2001). Anisakids larvae usually do not harm the fish because these are intermediate hosts or paratenic hosts (Rodríguez-Santiago *et al*., 2016). Suggesting that Pacific mackerel occupies an intermediate position in the food chain and may be part of the diet of many other marine species. Humans can become accidental hosts by ingesting anisakid-infected fish, which constitutes a potential risk to public health. Anisakid larvae are spread in highly consumed fish and have been associated with human infection after ingestion of raw or undercooked parasitized fish (Cruz *et al*., 2007; Rodríguez-Santiago *et al*., 2016).

Anisakids exhibit little or no specificity because they have been described in all oceans and in a wide variety of species of fish and cephalopods (Pérez-Ponce de Leon *et al*., 1999). The genus *Anisakis* is more abundant in temperate and polar environments (Anderson, 1984). Trematodes of the family Didymozoidae are common parasites of pelagic and oceanic fish (Pascual *et al*., 2006; Cruces *et al*., 2014). The highest diversity and abundance of marine Didymozoidae are found in the Pacific Ocean, with the region of Hawaii having the highest number of species (Nikolaeva, 1981). In this study Didymozoidae was the second group more prevalent and abundant. While the lowest prevalence and abundance in Cestoda (Tetraphyllidea) and Acanthocephala (*Rhadinorhynchus* sp.) were recorded in this study. It has been observed that the parasite populations vary according to the diet, host species, age-classes, geographic location, season and time. Overall, these dynamics rely on a latitudinal gradient of environmental conditions, influencing the distribution of zooplankton which, in turn, determines the differences in parasite assemblages among host populations. These assemblages are reinforced by the behaviour differences in the migration of hosts (Timi, 2003; Timi & Poulin, 2003).

On the other hand, the fish sampled during the two sample times were possibly a heterogeneous group of ages (1 to 3 years) with lengths ranging from 20 to 30 cm according to those reported by Knaggs & Parrish (1973) and Schaefer (1980). Espínola-Novelo *et al*. 2020 indicated that a seriated pattern of succession was detected in host species with a higher number of age-classes, which increased the probability of obtaining a more accurate estimate of the total number of parasite species that one host species can harbor along their ontogeny. Thus, the number of age-classes considered for a given species is more important than the geographic range. Therefore, independent of the geographic range, older fish achieved a balanced community with little change in parasite species (Price, 1990). However, many wild fish parasites may be scarce or absent, hence parasite cumulative infection is also scarce. Nevertheless, fish parasites have distinct quantitative and qualitative characteristics (George-Nascimento & Moscoso, 2013).

This is the first study on the parasitic composition and some seasonal variations (spring and summer) in abundance, prevalence, and intensity parasitic in the Pacific mackerel (*S. japonicus*) from Todos Santos Bay, Baja California, Mexico. Comparatively, with other fish species analyzed in other works in Baja California. Pacific mackerel had the highest prevalence and abundance of the nematodes (anisakid). Consequently, this shows the ecologic role of the Pacific mackerel to transfer this kind of parasite to other hosts species.

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Conflict of Interest

Authors state no conflict of interest.

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