FATTY ACID IN ECTOPARASITE COPEPODS FROM SOUTHERN CHILE *CALIGUS ROGERCRESSEYI* BOXSHALL & BRAVO 2000, *LEPEOPHTHEIRUS MUGILOIDIS* VILLALBA & DURAN 1985 AND THE FREE-LIVING SPECIES *TIGRIOPUS* SP

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ABSTRACT

This work presents the fatty acid composition of copepod species in southern Chile: two of them that are ectoparasites on *Eleginops maclovinus* (*Caligus rogercresseyi* and *Lepeophtheirus mugiloidis*) and one free-living species (*Tigriopus* sp.). *C. rogercresseyi* females from different hosts (*Salmo salar* and *E. Maclovinus*). Fatty acid methyl esters were determined with GC-MS.

The studied species presented a wide variety of saturated, monounsaturated, and polyunsaturated fatty acids, with compounds having from 12 to 24 carbons. The studied species had different percentage compositions of the acids identified. In all three species, palmitic (C₁₆) and oleic (C_{18:1}) fatty acids dominated the percentage concentrations. The highest percentage concentration (46.59 %) was found for palmitic acid in *Tigriopus*. Only the females of *C. rogercresseyi* analyzed were found to have myristoleic acid (C_{14:1}).

Stearic (C₁₈) and oleic (C_{18:1}) acids were present in both *C. rogercresseyi* males and females, but with different distributions. Oleic acid in females was 33.97 % and only 7.64 % in males, whereas stearic acid was 9.95 % in females and 21.51 % in males. The *C. rogercresseyi* on Patagonian blennie (*Eleginops maclovinus*) revealed 3.89% C_{20:4} and 9.60 % C_{20:5}. Eicosapentaenoic and docosahexaenoic acids, detected only in the ectoparasitic copepods, had percentage concentrations of up to 10 %. The possible role of this fatty acid in the stimulation of innate fish immunity is discussed.

Key words: fatty acids, ectoparasites, *Caligus rogercresseyi*, *Lepeophtheirus mugiloidis*, *Tigriopus* sp.

INTRODUCTION

The lipid content in marine zooplankton is of considerable interest due to the role it plays in life cycles, metabolic transformations, and trophic transfers¹. In terms of nutrition, its fatty acids constitute an interesting characteristic for aquaculture. Fatty acids are active in physiologic functions as components of biological membranes, forming part of the phospholipid and glycolipid. Moreover, they are combustible molecules and some of their derivatives act as hormones or messengers². The presence of long-chain polyunsaturated fatty acids, especially eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids is known to provide significant benefits for fish health. Fatty acids act in the mechanisms through which the lipid diet can intervene in the immune function of the fish and its resistance to illnesses³. The supply of DHA and EPA for the normal development of fish, shrimp, and mollusk larvae is known to be important in aquaculture. Microbial lipids, particularly polyunsaturated fatty acids but also monounsaturated and saturated fatty acids, have potential commercial applications in aquaculture as nutraceuticals, pharmaceuticals, and nutritional ingredients⁴. Crustaceans, especially copepods, are one source of these fatty acids in the aquatic environment⁵.

Copepods play an important role in zooplankton populations. They are one of the most important nutritional resources for fish and crustaceans, and are considered to be among the feeding alternatives for aquaculture. Many copepods are fish parasites. Specifically, the ectoparasites known as sea lice develop the stages of their life cycle on fish, generating great problems in salmon farming centers. In Chile, *Caligus rogercresseyi* is the dominant copepod ectoparasite affecting the salmon industry, causing serious damage to salmon farming in Chile⁶.

This parasite was transmitted to salmon farming centers by Patagonian blennie (*Eleginops maclovinus*, Cuvier & Valenciennes 1830)⁷, a native fish that has a wide geographic distribution off the Chilean coast, especially in the far-southern, southern, and central areas as far north as Valparaíso, including the coast of Argentina and the Falkland/Malvinas Islands⁸. The parasite fauna of this fish is mainly composed of two ectoparasites: *C. rogercresseyi* and *Lepeophtheirus mugiloidis*⁹. Of these, only the first affects salmon farming centers.

The control of these copepods, which are causing great damage in salmon farming centers, remains an unresolved issue. Some studies have set out to find factors to stimulate the immune response of the fish when faced with illnesses¹⁰. For example, Grayson¹¹ demonstrated that the immunization of the Atlantic salmon (*Salmo salar*) with a partially purified extract of *Lepeophtheirus salmonis* and of *Caligus elongatus* resulted in partial immunity against *L.*

salmonis copepodites. Other studies have considered the physiological and immunological relationship between *L. salmonis* and its host, indicating variations in the host's immune response when faced with an ectoparasite attack after being infected with it¹². Furthermore, studies indicate that ectoparasites produce compounds that limit the immune response of the affected fish¹³. An extract from the copepod *Calanus finmarchicus* is currently being studied to evaluate whether its use stimulates an immune response against ectoparasitic attacks in salmon; this investigation is being carried out by the company Skretting¹⁴. *C. finmarchicus* is also used for feeding fish¹⁵. Given the information available, it is interesting to analyze and identify the lipid components of the ectoparasites that attack fish farms and to compare these copepods with others used for feeding these same fish such as *Tigriopus*. This has been massively farmed and used for feeding fish and shrimp larvae^{16,17}. Due to the importance of fatty acids, the objective of this work is providing information leading to the control of copepod infestations, and the concept of using these fatty acids as immunostimulants against copepod attacks, we extracted, identified, and compared the fatty acid composition of the ectoparasite copepods *Caligus rogercresseyi* and *Lepeophtheirus mugiloidis* and the free-living copepod *Tigriopus* sp.

EXPERIMENTAL

Copepod collection

Caligus rogercresseyi samples were obtained from salmon cultures (*Salmo salar*, average size 60 cm) in La Arena cove, 50 km south of Puerto Montt (the farming center is the property of AquaChile). The samples were separated into males and females. *Lepeophtheirus mugiloidis* samples were obtained from *Eleginops maclovinus* (Patagonian blennie) caught in Angelmó Bay, off Puerto Montt. *Tigriopus* sp. was collected in the coastal area off Valdivia. The samples were collected between August and September, 2007. The species were identified by Gladys Asencio G., M.S., in the Centro I-MAR, Universidad de los Lagos, Puerto Montt.

Preparation of fatty acid methyl esters by direct transesterification

The identified samples were collected in tubes and frozen at -20°C for 48 hours¹⁸. Later they were lyophilized for 48 hours (water elimination). The samples were separated to extract the fatty acids in triplicate. Fifty milligrams of the lyophilized copepod samples were weighed and placed in 12-mL glass tubes with screw tops. A reaction mixture (3 mL; methanol: hydrochloric acid: chloroform, 10:1:1 v/v) was added to each tube. The sample was suspended in this solution by vortex agitation and was immediately incubated at 90°C for 60 minutes for transesterification. The reaction tubes were left to cool at room

temperature. After that, 1 mL of distilled water was added to each tube and the mix was agitated. Then, a fatty acid extraction mixture was added (hexane:chloroform, 4:1 v/v, 3 x 2 mL). The samples were centrifuged to separate the organic phase from the methylated fatty acids. Blank controls (i.e. containing no sample) were used in each experiment⁴.

Analysis of the fatty acid methyl esters using GC-MS

The fatty acids were identified as methyl esters using gas chromatography-mass spectrometry (Focus model, Thermo Electron Corporation, Waltham, USA) coupled with a mass spectrometer (DSQ model, Thermo Electron Corporation) equipped with a DBP-1 capillary column (30 m, 0.2 mm, 0.33 μ m). Helium was used as the carrier gas, with a flow of 1.5 mL/min. The mass spectrum obtained was between 35 and 500 m/z. Ionization was carried out by electronic impact at 70 eV. The detector temperature was 200°C. A 1 μ L sample in splitless mode was injected for each sample with the injector temperature of 250°C. The initial column temperature was 40°C, increasing 5°C/min until reaching 280°C and maintaining this temperature for 10 min¹⁹. The interface temperature was programmed at 300°C.

Identification of fatty acid methyl esters

The esterified fatty acids were identified by comparing the mass spectra with the NIST MS 2.0 library data (data included in Xcalibur™, the GC-MS software) and by determining the Kovats index, using a homologous series of n-alkanes, and comparing the indexes with values reported in standard reference data bases of the NIST²⁰. Methylated fatty acid standards (Sigma Aldrich) were also used in the identification.

RESULTS AND DISCUSSION

Table 1 provides a detailed description of the average percentages of esterified fatty acids identified. A total of 17 fatty acids with 12 to 24 carbon atoms were detected and identified in the three species of analyzed copepods; these were identified with the NIST MS 2.0 library, the Kovats index, and by comparing values with bibliographic data²⁰. Saturated and unsaturated fatty acids were found in all cases. Figure 1 shows the chromatographic profile of esterified fatty acids for female and male *Caligus rogercresseyi* from *S. salar* and *E. maclovinus*; females *L. mugiloidis* from *E. maclovinus* and *Tigriopus* sp. When comparing the saturated and unsaturated fatty acids, important differences were observed. Males of *C. rogercresseyi* had a higher proportion of saturated fatty acids (72.08 %) than the females did (41.81 %). *Tigriopus* presented the highest percentage of saturated fatty acids (85.24 %). The same fatty acids were dominant components in nearly all the copepods studied, which often are found in Crustacea. The most abundant saturated acid present in all three studied species, palmitic acid (C₁₆), was the highest in proportion in the free-living species (46.59 %). This very important acid is the precursor of the saturated and unsaturated fatty acids C₁₈, C₂₀, and C₂₂, all necessary for the diet of the organisms.

The comparison between males and females the *C. rogercresseyi* revealed differences in the composition: myristoleic acid (C_{14:1}) was only found in females independent of host (Figura 2).

In relation to the percentage composition, the largest difference was found between estearic (C_{18}) and oleic ($C_{18:1}$) acids. *C. rogerresseyi* females had 33.97 % oleic acid compared to 7.64 in males, whereas these same females had 9.95 % estearic acid compared to 21.51 % in males. These values are similar to those obtained from *C. rogerresseyi* females coming from Patagonian blennie. The comparison of fatty acids from the females of both ectoparasite species originating from *S. salar* and *E. maclovinus* (Table 1) did not present significant differences in terms of their percentage composition and distribution.

In *Tigriopus* sp., the free-living copepod, the majority of the identified acids were saturated (85.24 %). None of the unsaturated fatty acids found in analyzed ectoparasite copepods ($C_{14:1}$, $C_{20:2}$, $C_{20:3}$, $C_{22:6}$) were detected.

The most relevant fatty acids in marine fish and in the early developmental stages of mammals²¹, EPA and DHA, were found in interesting percentages, especially DHA, which exceeded 10 % in all cases except *Tigriopus* sp., in which it was not detected. In the case of the precursors of eicosanoids²², the fatty acids C_{20:3}^{14:7}, C_{20:4}^{20:4} and C_{20:5}^{20:1} only the latter two were detected and these were found in higher proportions in *C. rogercesseyi* from Patagonian blennies with 3.89 % C_{20:4} and 9.60 % C_{20:5}.

CONCLUSION

In aquaculture studies, highly unsaturated fatty acids, a subset of polyunsaturated fatty acids, have been found to be critical for maintaining high

growth, survival and reproductive rates and high food conversion efficiencies for a wide variety of marine and freshwater organisms. Polyunsaturated fatty acids play an important role in regulating cell membrane properties, serve as precursors for important animal hormones and are essential for animals. The dietary components of the fish include long-chain polyunsaturated fatty acids such as eicosatetraenoic, eicosapentaenoic, and docosahexaenoic acids that are present in copepods. The roll of these components in the fish this related to the energy production, components of greasy, component membrane weaves²³. Also these components are known precursors of immune system components. For example, the biologically active substance, eicosanoid, participates in the defense of organisms (prostaglandins, thromboxanes, leukotrienes, hydroxy acids), substances that influence a wide gamut of immune functions. These substances are derived through enzymatic reactions (cyclooxygenases and lipoxygenases) of polyunsaturated fatty acids with 20 carbon atoms²⁴.

In order to strengthen the immunological response in affected fish and to complement studies currently being carried out^{11,13}, these ectoparasites could be used to analyze the stimulation of innate fish immunity²⁵ since they have an interesting mix of saturated, monounsaturated, and polyunsaturated fatty acids, different to the fatty acids from free-living species, used frequently like food for the development of the fish.

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Figure 1: GC profile of esterified fatty acids present in *Caligus rogercresseyi*, *Lepeophtheirus mugiloidis* and *Tigriopus* sp.

A: *Caligus rogercresseyi* females from *Salmo salar*; B: *C. rogercresseyi* males from *Salmo salar*; C: *Lepeophtheirus mugiloidis* females from *E. maclovinus*; D: *Tigriopus* sp. males and females.

Figure 2: Comparison between males and females the *C. rogercresseyi* revealing differences in the composition of the myristoleic acid ($C_{14:1}$):

A: *C. rogercresseyi* females from *Salmo salar*; B: *C. rogercresseyi* males from *Salmo salar*; C: *C. rogercresseyi* females from *E. maclovinus*; D: *C. rogercresseyi* males from *E. maclovinus*.

Table 1: Fatty acid composition (fatty acid methyl esters) of *Caligus rogercresseyi*, *Lepeophtheirus mugiloides*, and *Tigriopus* sp., given as percentages (mean $\pm$ SD; n=3).

Fatty acid	R.T.	KI	<i>Caligus</i> 1	<i>Caligus</i> 2	<i>Caligus</i> 3	<i>Caligus</i> 4	<i>Lepeophtheirus</i> 5	<i>Tigriopus</i> 6
C ₁₂	22.58	1504	1.39 $\pm$ 0.38	4.09 $\pm$ 1.34	0.24 $\pm$ 0.02	0.03 $\pm$ 0.01	1.37 $\pm$ 1.52	3.07 $\pm$ 1.07
C ₁₄	27.13	1704	1.97 $\pm$ 0.07	4.92 $\pm$ 0.24	1.76 $\pm$ 0.15	1.99 $\pm$ 0.25	3.96 $\pm$ 1.59	10.13 $\pm$ 0.79
C _{14:1}	26.47	1686	0.84 $\pm$ 0.03	nd	0.74 $\pm$ 0.02	n.d.	1.38 $\pm$ 0.19	nd
C ₁₅	29.22	1804	0.15 $\pm$ 0.01	0.85 $\pm$ 0.06	0.17 $\pm$ 0.01	0.41 $\pm$ 0.01	0.30 $\pm$ 0.13	2.18 $\pm$ 0.16
C ₁₆	31.37	1911	27.45 $\pm$ 0.44	38.05 $\pm$ 1.60	18.64 $\pm$ 0.43	19.54 $\pm$ 0.48	22.29 $\pm$ 5.65	46.59 $\pm$ 3.37
C _{16:1}	30.80	1883	6.67 $\pm$ 0.30	2.22 $\pm$ 0.30	7.22 $\pm$ 0.54	4.84 $\pm$ 0.71	7.15 $\pm$ 1.09	6.37 $\pm$ 0.34
C ₁₇	33,14	2003	0.20 $\pm$ 0.02	0.52 $\pm$ 0.05	0.50 $\pm$ 0.01	0.83 $\pm$ 0.09	0.69 $\pm$ 0.17	2.04 $\pm$ 0.21
C ₁₈	35,06	2109	9.95 $\pm$ 0.58	21.51 $\pm$ 1.12	10.67 $\pm$ 0.26	13.51 $\pm$ 0.08	10.93 $\pm$ 2.95	13.16 $\pm$ 1.62
C _{18:1}	34.64	2086	33.97 $\pm$ 0.06	7.64 $\pm$ 0.17	25.78 $\pm$ 0.78	12.85 $\pm$ 2.85	24.19 $\pm$ 6.33	4.90 $\pm$ 0.53
C _{18:2}	34.29	2067	1.80 $\pm$ 0.17	3.04 $\pm$ 0.49	1.34 $\pm$ 0.22	1.95 $\pm$ 0.05	1.02 $\pm$ 0.19	1.00 $\pm$ 0.62
C ₂₀	38,43	2305	0.45 $\pm$ 0.06	1.05 $\pm$ 0.24	1.24 $\pm$ 0.22	0.93 $\pm$ 0.04	1.75 $\pm$ 0.62	1.63 $\pm$ 0.35
C _{20:1}	37,94	2278	1.34 $\pm$ 0.05	0.36 $\pm$ 0.05	2.32 $\pm$ 0.04	1.32 $\pm$ 0.04	1.41 $\pm$ 0.38	nd
C _{20:4}	37,10	2226	0.67 $\pm$ 0.06	0.44 $\pm$ 0.03	3.89 $\pm$ 0.85	2.84 $\pm$ 0.42	1.89 $\pm$ 0.70	nd
C _{20:5}	37,16	2230	2.01 $\pm$ 0.12	1.63 $\pm$ 0.08	9.60 $\pm$ 0.56	11.59 $\pm$ 0.68	5.19 $\pm$ 2.88	2.50 $\pm$ 0.03
C ₂₂	41,61	2505	0.25 $\pm$ 0.06	1.09 $\pm$ 0.25	0.27 $\pm$ 0.01	0.45 $\pm$ 0.09	0.49 $\pm$ 0.17	2.58 $\pm$ 0.65
C _{22:6}	40,17	2413	10.90 $\pm$ 0.74	12.58 $\pm$ 1.85	14.81 $\pm$ 0.01	25.16 $\pm$ 1.33	14.75 $\pm$ 2.20	nd
C ₂₄	44,55	2699	nd	nd	0.86 $\pm$ 0.01	1.79 $\pm$ 0.08	1.24 $\pm$ 0.55	3.86 $\pm$ 1.03
SFA			41.81	72.08	34.35	39.58	43.02	85.24
MUFA			42.82	10.22	36.06	19.32	34.13	11.27
PUFA			15.38	17.69	29.64	41.32	22.85	3.5

KI: Kovats index; 1: *Caligus rogercresseyi* females from *Salmo salar*; 2: *C. rogercresseyi* males from *Salmo salar*; 3: *C. rogercresseyi* females from *Eleginops maclovinus*; 4: *C. rogercresseyi* males from *Eleginops maclovinus*; 5: *Lepeophtheirus mugiloides* females from *E. maclovinus*; 6: *Tigriopus* sp. males and females; nd: not detected

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid

R.T.: Retention time

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